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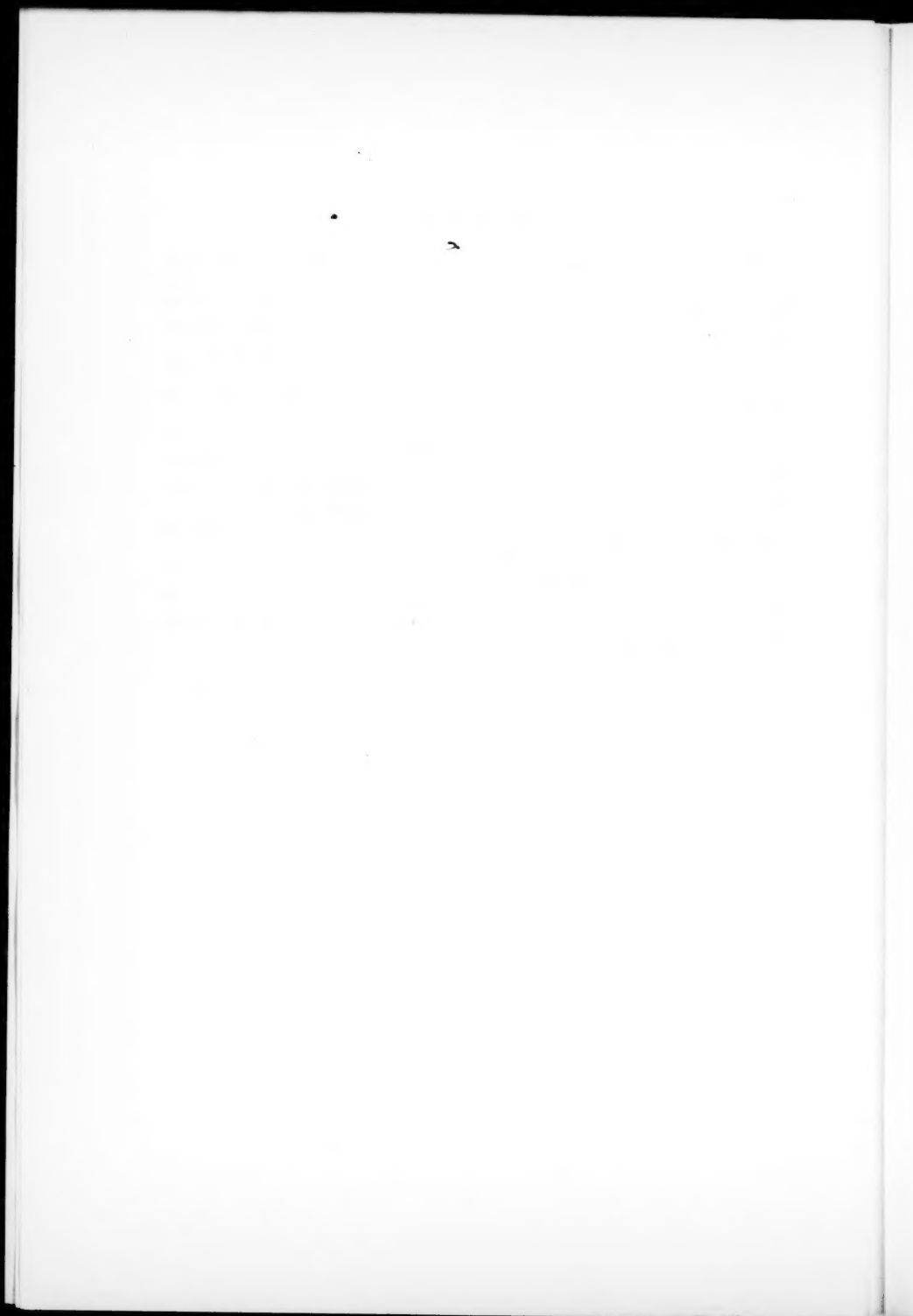*University of California*

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PREFACE

The Preface to this *Review* provides another opportunity for us to express our thanks to those who have prepared the chapters for this volume. We feel that their surveys of the current literature in widespread fields of microbiology are important contributions to a more complete understanding of microorganisms and their behavior in the test tube or in their natural environments. The Editorial Committee endeavors to provide surveys of different fields as they develop, and it greatly appreciates suggestions for future topics and authors.

We wish to extend our thanks to Dr. Wallace E. Herrell, who is retiring this year from the Editorial Committee, and to welcome Dr. Perry Wilson as a new member. Dr. Herrell has graciously contributed much time and effort to the establishment and development of this *Review*. We announce with regret the resignation of Dr. H. A. Barker as an associate editor and extend to him our appreciation of his many services.
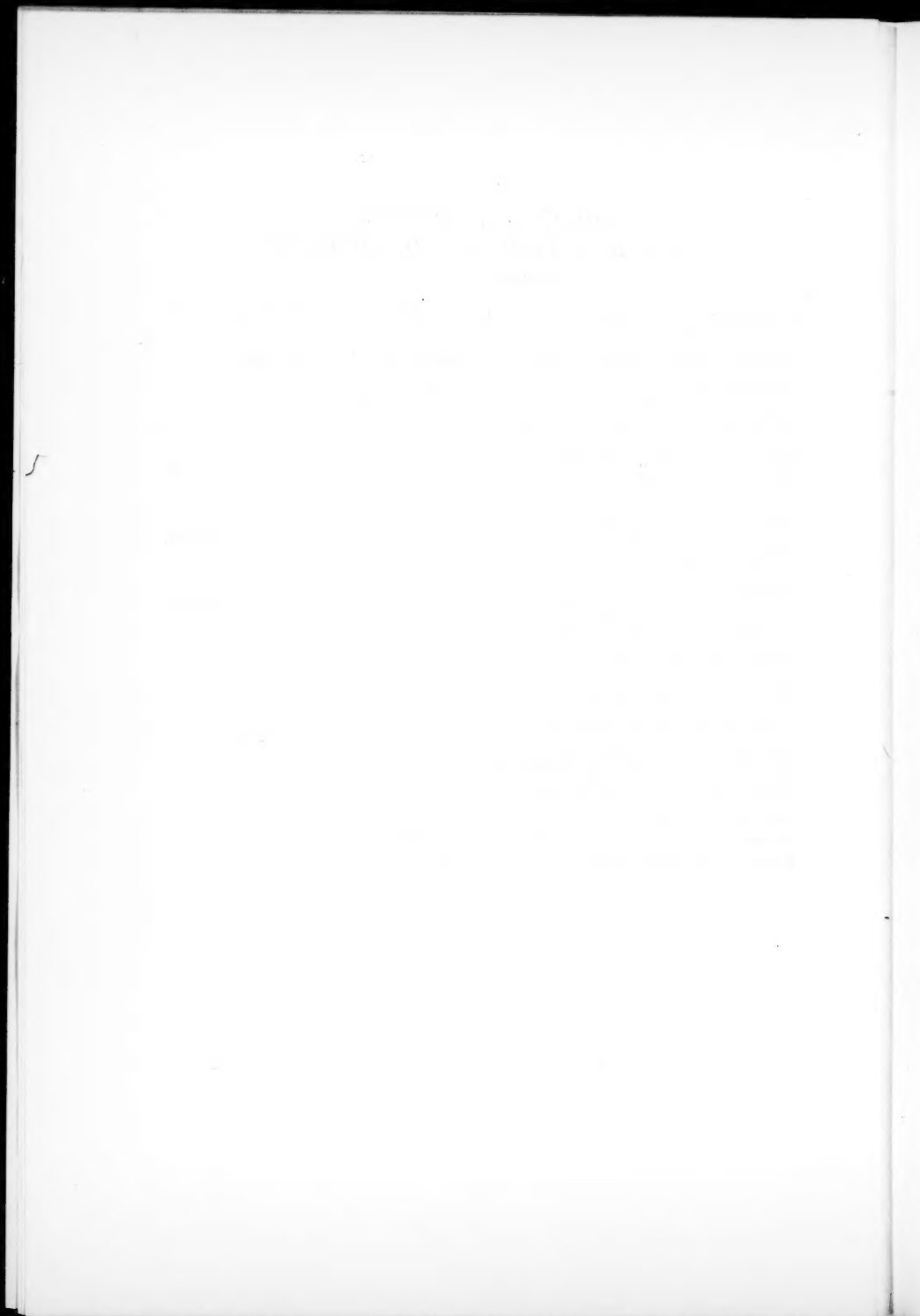
Once again it is a pleasure to acknowledge our deepest appreciation for the kind assistance given us by the office staff of Annual Reviews and for the painstaking care exercised by them and by the George Banta Publishing Company in the preparation of this volume.

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TOPICS AND AUTHORS
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MORPHOLOGY OF PROTOZOA[1,2]

BY E. FAURÉ-FREMIET[3]

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Morphology of the protozoa is such an extensive subject that it is impossible to offer here even mention of all pertinent work which has appeared since the review given by Wenrich (163) in 1947 and by Sonneborn (135) in 1949. The present paper arbitrarily will be restricted to consideration of the following three topics: cytological aspects of the organization of protozoa; comparative morphology and evolution of flagellates and ciliates; mechanisms of morphogenesis in ciliates.

CYTOLOGICAL ASPECTS OF PROTOZOAN ORGANIZATION

Chromosomes.—In spite of the difficulties frequently encountered with protozoa when one attempts to compare the morphological constituents of nuclei, which themselves are of structurally different types, a precise understanding of the chromosomes has been gained in at least several groups.

In a few flagellates the small number and large size of the chromosomes, e.g., about 50μ long in *Holomastigoides tusitala* (26), permit a detailed study of their structure. Their permanence during interphase has been established by Grassé (74, 75) and Cleveland (26). The chromonema is embedded in a matrix; a minor coil persists throughout the life cycle and duplication occurs during very early prophase; a major and supercoil are superimposed upon the minor coil during mitosis and, especially, meiosis (26). *H. tusitala* possesses two giant chromosomes, one short with a terminal nucleolus, the other long with one lateral and one terminal nucleolus (26). *H. rosei* possesses four, each in the form of a V with unequal arms; their structure is intercalated or atelomitic, with a nucleolar organizer at the level of a constriction (75). The cycle of duplication of these giant chromosomes can occur, independent of nuclear division, by endomitosis; Cleveland (26) has counted two, three, or four chromosomes in *H. diversa*.

The fate of chromosomes during mitosis and meiosis in a large number of species of flagellates parasitic in the wood-feeding roach *Cryptocercus punctulatus* has been traced in detail by Cleveland in a series of eight papers, the first and last of which are cited here [Cleveland (27, 28)], on the effects of the molting hormone of the host on the sexual cycles of the flagellates.

The haploid nucleus of several of the Foraminifera, e.g., *Patellina cor-*

rugata, shows 24 large, spiralled, atelomitic chromosomes [Le Calvez (105, 106)]. Among the Opalinida, the investigations by Chen [reviewed by Sonneborn (135)] showed that the chromosomes of *Zelleriella* are comparable in detail with the chromosomes of higher organisms.

The problem of chromosomes appears much more difficult among the Euciliata (38, 135). The latest researches of Devidé (37) confirm the fact that the "chromosomes" often described in the course of vegetative mitoses of the micronucleus are actually chromosome aggregates whose apparent transverse division, as well as inconstancy of number and form, does not contradict modern karyological ideas. In *Colpidium campylum* very small chromatic granules are observable at prophase; their fusion into irregular meridional bands occurs at metaphase; during telophase constriction and division of the bands take place followed by their reconversion into fine granules. True chromosomes appear, only during meiosis, in the form of very fine filaments showing some aspects of pairing, followed by the formation of tetrads; these same aspects have been described by Grell (77, 78) for *Ephelota*. In the course of the postmeiotic, then metagamic and somatic (or vegetative) divisions, the true chromosomes will be masked progressively by the development of an abundant matrix and by nucleolar substances which give the effects of aggregation.

The microspectrophotometric determinations of Moses (114) show that the micronucleus of *Paramecium* contains a large proportion of ribonucleic acid; viz., for 87.6 per cent total protein, 8.2 per cent is of RNA[2] and 4.2 of DNA.[2] Seshachar (131), using a similar technique with the gymnostome *Chilodonella*, has established the occurrence of DNA synthesis after mitosis at the beginning of interphase, although duplication of chromosomic ribbons takes place at prophase in the case of the hypotrich *Urostyla* [Raabe (128) and Sonneborn (135)].

It is known that the macronucleus is formed after conjugation by the growth of one of the micronuclei arising by postzygotic divisions of the synkaryon; we are also aware that the macronucleus, justifiably considered a somatic nucleus, remains genetically active. Actually in a number of species or races of ciliates which have lost their micronucleus [Corliss (31, 32, 34) has described some new cases and has cited literature on the subject], the macronucleus alone controls the normal development of succeeding generations; and Sonneborn (132, 133) has demonstrated experimentally that it controls specific characters in *Paramecium*. Furthermore, a small fraction of the macronucleus suffices to reconstitute a functionally active macronucleus; this presupposes juxtaposition of numerous identical genomes in the initial macronucleus. The macronucleus is thus a polyenergid nucleus in the sense of Hartmann, polyploid in the sense of Piekarski and Geitler [cf. (120) and (133, 134, 135)].

Grell (77) has observed the first signs of polyploidization, in *Ephelota gemmipara*, when the macronuclear anlage begins to enlarge itself in the exconjugants. The endomitotic process manifests itself by repeated longi-

tudinal splitting of pairs of chromatids; the volume of karyolymph increases, and the chromosomes group themselves in contact with the heterochromatic chromocenters; their multiplication seems to continue, and the karyolymph is progressively invaded by granules or short rods, containing highly polymerized DNA, among which are located the "oxygranules" of Raabe (129) which Mugard (115) has homologized with true nucleoli because of their RNA content.

The composition of the macronucleus is comparable with that of the micronucleus in so far as that for 85.1 per cent total protein, 9.1 is of RNA and 5.8 of DNA (114). Electron micrographs of the macronucleus of *Paramecium* (16) and of *Spirostomum* (70) reveal the presence of oval particles and of spheroids, measuring about 150 mμ for the former and 200 to 300 mμ for the latter, corresponding to DNA-containing granules, and of globules measuring 1μ or more corresponding to nucleoli. Do the DNA-containing granules correspond to chromosomes? The question remains problematical, like the interpretation of the chromatic bands of the micronucleus in vegetative mitosis. The behavior of the "reorganization bands" in the macronucleus of hypotrichs [Kiesselbach (92)], which allows an assumption of reversible changes in the state of dispersion or aggregation of submicroscopic DNA-containing particles, poses a still graver problem. Such particles could become united in a viscous mass or in vacuolized droplets, the aspect of which, as figured by Kudo (103) for *Nyctotherus*, calls to mind a state of coacervation [Bungenberg de Jong (19)]. Do chromosomes, properly speaking, exist in the normal macronucleus in the form of ultramicroscopic units?

Concerning the polyploid character of the macronucleus, Sonneborn (133) wrote: "The gross amitotic division of the macronucleus is merely a segregation of one group of discrete and genetically complete subnuclei into two groups." Nuclear budding in the Suctoria leading to successive isolation of small identical nuclei, e.g., in *Tachyblaston*, is considered by Grell (78) as a "depolyploidization" isolating a series of complete genomes. This does not mean that each subnucleus is exactly reduced to a single diploid set; it is known, from the work of Sonneborn (132), that the micronucleus of *Paramecium aurelia* is insufficient for controlling the phenotype.

The properties of the macronucleus can vary with time and in space during the vegetative period. Weisz (153, 156, 158, 159) discovered that, in *Stentor coeruleus*, the colorability of the macronuclear nodes by methyl green diminishes following an anteroposterior intensity gradient and that their "morphogenetic potential" decreases in a parallel manner. The degree of polymerization of DNA [or the extent of its association with proteins; cf., Alfert (1)], indicated by its colorability, will, therefore, be important in the control of morphogenesis. The fusion of nodes before division regenerates the equipotentiality of the macronucleus [cf. Brachet (14) for the role of the nucleus in processes of synthesis].

Among different Foraminifera the nucleus of the gamont hypertrophies and then degenerates by a karyolysis which liberates a micronucleus. De-

scribing this process for *Iridia*, Le Calvez (105) interpreted it as segregation of euchromatic material and of the genome, comparable with production of the female pronucleus after the formation of the germinal vesicle.

Mitosis.—The nucleus of a trichomonad, *Caduceia theobromae*, shows, during interphase, the chromosomes bound by a "chromodesmose" at their centromeres which are arranged in contact with the nuclear membrane [Grassé (74)]. The centromeres divide at prophase while the chromosomes, disposed in a bouquet, cleave longitudinally; the two groups of reduplicated centromeres then glide along the membrane in separating the coupled chromatids. This type of mitosis, which is found in the trichonymphids and spirotrichonymphids, is characterized, according to Grassé, by the dynamic role of the centromeres fixed upon the nuclear membrane rather than upon an achromatic spindle. Cleveland (26) noted this same role of the centromeres during mitosis and, especially, meoisis in *Holomastigoides*. The general significance of these facts led Grassé (74, 75) to consider the multiple aspects of nuclear division among protozoa as reducible to two fundamental types: orthomitosis and pleuromitosis.

Orthomitosis is characterized by the spindle insertion of the centromeres and by the equatorial plate of the metaphase stage; classically known in the metazoa, it is found among the protozoa with different details, which Grassé has characterized after the names of genera or groups which show them: *Vahlkampfia*, *Pelomyxa*, *Chilomonas*, *Euglena*, *Entamoeba*, *Euglypha*, the Opalinida, the Sporozoa.

Pleuromitosis has just been described above for a trichomonad; the centromeres are not inserted directly on the spindle, and metaphase (strictly speaking) is missing. These characters throw light on the mechanism typical of mitosis in the Dinoflagellata and on the controversial interpretation of it in that group [Hovasse (86)]. In *Syndinium turbo* in the course of this type of mitosis, which is termed "syndinienne" by Chatton, 5 chromosomes in a V, the point of which bears the centromere, converge in a bouquet with ten branches. Division of the centromeres, cleavage of the chromosomes, and progressive spreading apart of the two groups of centromeres separating the sister chromatids until they are located end to end before becoming disjoined, are undergone during the process of the so-called "caducéienne" or "trichonympheenne" mitosis. But in the case of *Noctiluca* or *Blastodinium crassum* the dinomitosis is complicated by a large astral figure whose significance remains undetermined. In the foraminiferans *Patellina* and *Iridia*, Le Calvez (105) figured some remarkable schizogonic dinomitoses, the "plaques centrosomiennes" of which he later (106) has interpreted as "plages centomériennes" following the suggestions of Grassé (74) who included this mode of division under pleuromitosis (75).

The division of the micronucleus of the Euciliata appears as an acentric orthomitosis, characterized at metaphase by the unique barrel-shaped appearance of the equatorial plate, that is to say, by the elongation of the chromatic strands on the spindle. If, as has been considered above, these

"strands" do not correspond to true chromosomes but to aggregates of chromosomes, it could be supposed that each of them bears a series of centromeres which would assure the longitudinal insertion of the entire ensemble on the spindle. The genetic paradox of a transverse division of the chromosome aggregates disappears if one accepts with Sonneborn (135) the interpretation of Raabe (128). In *Urostyla*, for example, the prophasic vegetative micronucleus contains a spireme already reduplicated, the turns of which split apart at metaphase and become elongated at the middle region of the spindle; at anaphase the longitudinal halves slide in opposite directions toward the contrary poles, the last stage giving the illusion of a transverse division. (The writer some 43 years ago published a correct drawing of this process but a completely wrong interpretation of it.)

In the course of the meiotic divisions Grell (77) and Devidé (37) observed numerous fine chromosomes in loops and in typical tetrads at the periphery of the spindle; at metaphase these elements were reassembled in the middle region of the spindle without forming a true equatorial plate (*Ephelota, Colpidium, Euplotes*).

The meiotic prophase in *Colpidium campylum* (37) shows the curious crescent stage already known for *Paramecium;* Corliss (31, 33) has observed at the onset of the autogamous cycle in *Tetrahymena rostrata* a comparable stage, characterized by a remarkable elongation of the micronucleus so that it curves itself in the form of a "hunting horn" as much as one and one-half times around the macronucleus. The almost inscrutable internal structure has not permitted an interpretation of this enigmatic stage.

Cytoplasm.—Since the reviews of Beams & King (9) and of MacLennan (112), a general well-documented survey on the cytoplasm of protozoa has been offered by Grassé (75); a treatment of the chondriome and various cytoplasmic inclusions may also be found in a number of recent investigations (20, 79, 115, 153, 157). An effort is being made today to supplement the information obtainable by the classical cytological techniques by application of phase contrast and electron microscopical methods to the study of protozoa.

Bairati & Lehmann (5, 6) and Lehmann (107) have revealed the presence of dispersed submicroscopic globules and filamentous proteins in the fluid and unstable sol phase of the hyaloplasm in *Amoeba proteus;* after desiccation some granular filaments are shown in electron micrographs. The gel phase appears as a three-dimensional, interlacing network of rows of submicroscopic granules each measuring about 100 mμ. Perhaps these can be separated out *in vitro* by centrifugation.

The electron micrographs of very thin sections of *Opalina* and *Paramecium* obtained by Bretschneider (16) reveal, side by side with the mitochondria, some endosomes of various sizes possibly corresponding to Golgi bodies (70) and some very fine granules corresponding to the microsomes of metazoan cells. These granules, like the metazoan microsomes, are perhaps of a diverse nature; employing dark-field, centrifugation, and microchemical

tests, Mugard (115), studying theronts of species of *Ophryoglena*, distinguished ribonucleoprotein microsomes and glycogen particles.

The pigment granules of *Stentor coeruleus* contain phospholipids, and Weisz (153, 157) believed they can be identified as mitochondria. In *Holosticha rubra* the reddish orange pigment has been extracted by Giese (73) with polar organic solvents.

Auto-reproducible particles and symbionts.—The centrosomal origin of the basal granules of cilia and flagella (kinetosomes, blepharoplasts), following the law of Henneguy-van Lenhossek, has been recognized for a number of protozoa. Chatton, Lwoff & collaborators, furthermore, have shown that these basal corpuscles are endowed with genetic continuity and can multiply themselves by division or budding in the absence of the centrosome. The general significance of this fact has been pointed out and discussed by Lwoff (109, 110) and Sonneborn (136).

It is possible, even probable, that the ribonucleic microsomes revealed in the cytoplasm by electron microscopy and centrifugation are themselves also autoreproducible; this character links them, at the same time, to microscopic cellular organites such as the plastids and to submicroscopic particles such as the plasmagenes and viruses. The kappa factor responsible for the killer character of certain strains of *Paramecium aurelia* [Sonneborn (135)] is also a self-duplicating, particulate entity; its rate of multiplication can vary from that of the paramecium itself under certain thermal conditions; it can be inhibited by x-rays and antibiotics; finally, this particle of microscopic size is revealed by DNA-specific stains (124, 125). This group of characteristics allows it to be considered either as a plasmagene or as a parasite, nonpathogenic for its usual host, such as a large virus or a rickettsial organism (134 to 138, 140).

The presence of bacteria associated with protozoa as symbionts has often been noted [cf., Kirby (93)]; among the ciliates the cytoplasm of several species of *Remanella* contain some Feulgen positive bodies reminiscent of kappa particles [(59) and Dragesco, unpublished]. In the case of several species of *Euplotes* the cytoplasm regularly contains some bacteria-like bodies dispersed among the mitochondria. Their disappearance, as effected by weak doses of penicillin, by cessation of multiplication of the ciliate, allowing the presumption that they exist as a necessary symbiont (62). One is also reminded of the dorsal covering of caulobacteria which characterizes the genus *Centrophorella* (58, 59, 61).

Cilia and flagella.—The electron microscope demonstrates with clarity the structural complexity of cilia and flagella which ordinary microscopic observation has always allowed us only to assume. We now know, for example, that a cilium of *Paramecium* or *Frontonia* (90, 130) is a tuft which, by treatment preceding desiccation, can be dissociated into eleven parallel fibrillae, apparently homogeneous, 30 to 50 mμ in thickness, one among them longer than the others; if the integrity of the fibrillar bundle is maintained by a suitable fixative, the cilium gives the contradictory picture of a single, thick, and homogeneous fiber.

According to Brown (17) the flagellar structure in *Euglena* and *Astasia* appears more complex, with an axoneme formed by two dense fibrils enclosed by a thinner sheath formed by, or containing, a helicoidal fibril and bearing delicate lateral filaments ("flimmer") whose exact nature is debatable. Brown (18) has also reported the presence of flimmer in *Chilomonas*. Bretschneider (15, 16), examining the cilia of *Opalina* and of the true ciliates *Isotricha* and *Paramecium* by electron microscopy, using sections 0.3 to 0.6 μ in thickness, described an analogous structure with an axial fibril 25 mμ thick surrounded by a protoplasmic sheath 30 mμ thick and by a membrane containing a spiralled fibril 25 mμ in thickness. Pitelka (123), however, has not verified the presence of a spiralled fiber on the flagella of *Euglena* and *Astasia* and has shown that the double axoneme is composed of about nine fibrils, with thicknesses of 35 to 60 mμ, grouped into two compact parallel bundles; the ensemble is enclosed in a semifluid sheath containing disorganized fibrillar elements which give the appearance of lateral mastigonemes when liberated by rupture of an externally limiting membrane.

According to Kleinschmidt & Kinder (99), the flagellum of trypanosomes is a bundle of parallel fibrils, ten to twelve in number. Likewise the cilia of *Paramecium*, after rapid desiccation, revealed to Krüger & Wohlfarth-Bottermann (102) a fibrillar bundle without helicoidal structure; and very thin transverse sections have shown one axial fibril surrounded by nine fibrils disposed in a circle within a sheath [Hamilton, Gettner & Stock (84)].

In résumé, cilia and flagella are basically composed of a bundle of elementary fibrils embedded in a matrix or enclosed in a sheath itself more or less differentiated. Should these fibrils be considered comparable with the flagella of the bacteria? The latter, examined *in situ* by electron microscopy (85), or even in suspension *in vitro* (4, 147, 148), are protein filaments about 12 mμ in thickness; their x-ray pattern shows the α-form reflection characteristic of fibrous proteins of the keratin-myosin class, and Astbury has thus been able to compare them with "monomolecular muscles."

The electron micrographs of ultra-thin sections of opalinids and ciliates also show the basal corpuscles of the cilia, the accessory granules, and the longitudinal uniting fibrils (15, 16, 102).

Cytoskeleton and cuticle.—The endoskeleton of the astome ciliates *Radiophrya* and *Maupasella* contains a group of rigid, elastic, and birefringent fibers made up of sulfur-containing scleroproteins [de Puytorac (126, 127)]; thus, it is similar to the skeletogenous substance stiffening the sucker or attaching disc of the Urceolariidae. *Coleps* possesses a tegumentary, mineralized exoskeleton, one-half of which is reconstituted after each fission; the calcification depends upon an enzymatic mechanism which can be inhibited by benzene-sulfamide (68).

Ectoplasmic pellicles may be classified into different types depending upon what species are examined. Using micromanipulation and the action of different salts, Pigoń (121) showed that the pellicle of *Opalina ranarum* overlays an ectoplasmic stratum rich in lipids. The stability of the system was found to be dependent upon the ionic composition of the medium. In

the case of *Paramecium*, the thin pellicle proper, containing the basal granules of the cilia and the underlying fibrillar system, can be dissociated from the overlying "pellicular lattice" which is dissolved *in vivo* by ammonium oxalate (122).

Trichocysts.—Since the discovery (89, 90) of the periodic structure of the explosive trichocysts of *Paramecium* [Wenrich (163)], new work employing dark-field, phase contrast, and electron microscopy has allowed completion of the research by Krüger (101), and recognition, among the ciliates, of four principal structural types.

The trichocysts of the *Paramecium* type ("Spindel Trichocysten" of Krüger) are widely distributed among the holotrichs. [Recent papers on *Paramecium* itself include (44), (100), (119), (165), (166); see also review of the literature in Wichterman (164).] The transverse striation characteristic of the ejected filament has been found again, with the same period of about 55 mμ, in three species of *Frontonia* (11, 102), in *Disematostoma*, and in the gymnostome *Nassula* (41, 42). Comparing the exploded trichocysts in seven species of *Paramecium*, Dragesco & Beyersdorfer (44) found exactly the same periodicity, the specific differences appearing only in the dimensions of the filament and in the form of the apical piece. Very recently Dragesco (43) has found some trichocysts of the same type in the dinoflagellate *Oxyrrhis marina*. On the other hand, the cryptomonad flagellate *Chilomonas paramecium* appears to possess two kinds of explosive trichocysts which differ from the *Paramecium* type [Dragesco (40)].

The significance of the periodic structure and its comparison with that of collagen fibrils remains a matter for hypothesis (10). Wohlfarth-Bottermann (166) has attributed the super-period visible in some images to the presence of an external helical fibril, an hypothesis not confirmed, however, by the shadow-casting technique. An hypothesis on the physiological role (fixation of calcium ions) of these nontoxic organites was inquired into by the same worker (165).

The toxic trichocysts ("Nesselkapselähnlichen Trichocysten" of Krüger) eject, by a sudden evagination, a long and more or less complex filament which is the vector of the toxic substance. Such trichocysts have been described by Krüger for a number of predaceous gymnostomes; their unique structure has been confirmed by Dragesco (42) using electron microscopy. The cytological effects immediately provoked by contact with the exploded organites [Canella (20)] have been nicely recorded in microcinematography by Dragesco & Métain (46).

The mucoid trichocysts, often described as "protrichocysts," are spindle- or rod-shaped subcuticular bodies which eject to the exterior a sticky, structureless substance; Dragesco (41) has found them in many ciliates and has compared them with the muciferous bodies of the euglenoid flagellates.

The "paratrichocysts" of Klein, distributed between the ciliary rows and occasionally filled with pigment (e.g., in *Stentor coeruleus*), were compared by Weisz (157) with mitochondria, but this remains debatable.

Hovasse (86) has contributed a new interpretation for the autogenesis of the nematocysts in the dinoflagellate *Polykrikos;* he (87) also has described some curious "discobolocysts" in a chrysomonad flagellate.

Tentacles of the Suctoria.—Study of the tentacles of the suctorian *Discophrya piriformis* by phase contrast and electron microscopy [Dragesco & Guilcher (45); Blanc-Brude, Dragesco & Hermet (12)] has clearly revealed structures of the central canal and of the cytoplasmic sheath. The latter bears small, enigmatic appendages and appears to fold upon itself in a spiral during contraction of the tentacle. The passage of ingested particles through the canal has been recorded by the technique of microcinematography.

COMPARATIVE MORPHOLOGY AND EVOLUTION

General works.—A recent volume of the *Traité de Zoologie*, edited by Grassé (143), is dedicated to the protozoa in general and to the flagellates in particular. Grassé's (75) chapter, "Généralités," includes a well-documented revision of our understanding of the protozoa; the place of these organisms in the world of life and the problems of their origins and of their relationship to the metazoa are clearly discussed, while leaving little doubt concerning the value of hypotheses which have been proposed for the resolution of such matters. The fundamental unicellularity of the protista is essentially defined by taking into account its diverse aspects, illustrated with several characteristic examples, from the first appearances of differentiation in a microorganism potentially unlimited in its development to a highly evolved form with a perishable soma.

In regard to comparative morphology, Grassé has arranged the protozoa in five sub-branches; namely: Rhizoflagellata (Flagellata + Rhizopoda proper); Actinopoda (Acantharia + Radiolaria + Heliozoa); Sporozoa; Cnidosporidia; Ciliata. The practical significance of this new presentation is stressed in the introduction to the section on the rhizoflagellates.

The third edition of Kudo's (104) excellent *Protozoology* has been reprinted; a greatly needed advanced text in protozoology, written by Hall (82), has just appeared; Jahn & Jahn (88) have outlined a general description, clearly illustrated, of all the protozoa; Wichterman (164) has published a very important monograph on the genus *Paramecium;* Lwoff (110) has exposed the particular problems posed by the mechanisms of morphogenesis in ciliates; moreover, he has edited (111) the first volume of a series of studies on the biochemistry and physiology of protozoa which goes beyond the subject of the present review although contributing to it important complementary information. Techniques applicable to the study of protozoa have been gathered together into a very useful manual by Kirby (95); those which apply especially to *Paramecium* have been published by Sonneborn (139). Various problems of morphogenesis in the protozoa in general and in the ciliates in particular have been treated in a book by Bonner (13) and in the symposia edited by van der Klaauw & Barge (96) and by the Centre National

de la Recherche Scientifique (29). We shall restrict ourselves in this review to works published recently concerning the comparative morphology of the flagellates and ciliates.

Flagellates.—On the basis of comparative morphology, the phytoflagellates, characterized by their power of photosynthesis, cannot be separated from the zooflagellates, and the *Traité de Zoologie* (143) accords them a large section. They show structural types diverse enough to justify their subdivision into some ten classes: Volvocales or Phytomonadina, Xanthomonadina, Chloromonadina, Euglenoidina, Cryptomonadina, Dinoflagellata, Ebrideae, Silicoflagellata, Coccolithophoridae, Chrysomonadina. Chatton, Deflandre, Hollande, and Pavillard have presented well-documented chapters on these groups.

The class of zooflagellates is subdivided into numerous orders, an expression of the remarkable diversification which has arisen from several basic patterns of organization. Kirby (94) and Grassé (76) have examined the general characters of evolution in this group on the basis of the structure of the "mastigont system" or "centro-blepharo-flagellar complex" (the "cinétide" of Chatton), the nucleus, and the mechanisms of division.

The environment provided by the rectum of cockroaches and xylophagous termites seems to have facilitated the astonishing evolutionary diversification of the Trichomonadida and the Hypermastigida. The principal mechanisms of this evolution are hypertelic development of structures, and the "polyisomerism" (Kirby) resulting from the multiplication of the structural units. For example, a *Stephanonympha* with its multiplication of karyomastigonts appears like a colonial devescovinid, although the individuality of the ensemble, attested to by the definite disposition of the morphological units, the anterior localization of the flagella, and the assemblage of the axostyles into a unified organite does not permit consideration of it as a "somatella" (76). In the case of *Calonympha* or *Snyderella* the potential independence of the self-reproducible organites, recognized some time ago by Janicki (91) and Chatton (21), manifests itself by the regular and orderly coexistence of karyomastigonts and akaryomastigonts; that is to say, by asynchronism in the division of the nuclei and, more rapidly, the kinetoflagellar elements.

The Hypermastigida are derived from the Trichomonadida by a more marked dissociation, such that the repeated duplications of the kinetoflagellar organites, independent of all immediate relation to the poles of the spindle, lead to polyisomerism (94) of the mastigont type of organization, while the nucleus remains simple. The mechanisms of division, therefore, can be modified so that either the highly differentiated flagellar organites are renewed entirely in the course of the process (Lophomonadina) or they are divided between the two sister cells, each of these then replacing the missing parts.

Grassé (76) has described for these groups a highly ramified evolution, each branch of which is an orthogenetic line unique in its manner of multiplication of the blepharoplasts, in the complexity of the centrosome, in the

hypertrophy of the parabasal body, in the tendency for gigantism, and in the complication of structures with even disappearance of some, such as the cresta.

Opalinids.—For some time now considered by a number of protozoologists as flagellates rather than "protociliates" [e.g., Gatenby & King (72); Chatton & Brachon (22); and cf. Grassé (143)], the opalinids are characterized by the absence of a centrosome. This entails complete disjunction between the nuclei and the kinetoflagellar apparatus during acentric mitosis as well as the loss of all the organites bound to the centrosome in the Trichomonadida and Hypermastigida. The unity of the kinetoflagellar ensemble, however, is shown by an apico-ventral line of kinetosomes, double or multiple, from both sides of which the longitudinal ciliary rows are formed and depart. This apico-ventral line is the *cinétie génératrice* of Chatton & Brachon (22) and the falx of Mohr (113); it is cut transversely in the course of the longitudinal fission, separating intact ciliary rows into two groups. Cosgrove (36) and Fernandez-Galiano (69) have given precise details of this very particular organization which may be considered as derived from that of the Flagellata.

Ciliates.—Use of various techniques of silver impregnation, which reveal ciliary patterns with extreme precision, has contributed some new facts of great importance in interpretation of the comparative morphology and evolution of the ciliates. One will find accounts and discussion of these in the publications of Klein (97, 98), Chatton & Séguéla (25) and Lwoff (109, 110). A brief technical description of the Chatton-Lwoff refinement of the original Klein "dry" method, the modification customarily employed in the writer's own laboratory, has recently been published in English [Corliss (35)].

Like the opalinids the ciliates are characterized by the absence of a centrosome; but their ciliary rows (kineties) behave as so many independent morphological units; they grow in length by multiplication of the kinetosomes; binary fission (type *percinétienne*) cuts them transversely and distributes the halves, in equal numbers, between the two homothetic systems which are the daughter cells. The structural differentiation of the ciliature, however, can express itself in such dissymmetry that to realize a condition of homothety the mechanisms of division must be completed by the most complicated processes of morphogenesis.

The study of thigmotrich ciliates carried out by Chatton & Lwoff (23, 24), following their earlier researches on the apostomes, stands as another noteworthy example of what can be expected from comparison of ciliary patterns and of the mechanisms of their morphogenesis for gaining an understanding of poorly known groups. Evolution of these ciliates, following several orthogenetic lines, is dominated by the anterior localization of the thigmotactic area, the more and more accentuated removal posteriorly of the ciliary buccal system and the autonomization of the latter (*Hemispeira*). The adaptation to parasitism involves a reduction of the ciliature and the appearance of a new organite: the sucker of the Rhynchodea.

Furgason (71) first accurately defined and recognized the significance of

the tetrahymenal buccal ciliature of ciliates in the *Colpidium-Glaucoma-Leucophrys-Tetrahymena* group [cf. Corliss (30, 31, 32, 34)]. This structural type, whose morphogenesis was also studied by Chatton & colleagues (24a), is very widely distributed among the hymenostome holotrichs; Mugard (115) showed how it is variously modified in the orthogenetic lines of the Ophryoglenidae, the Philasteridae and the Lembidae. In the cases of *Frontonia*, *Disematostoma*, and *Stokesia*, on the other hand, one finds (55) the "peniculi" and the "membrana quadripartita" described by von Gelei (72a) for *Paramecium*; because of this, notions concerning trichostome and hymenostome holotrichs must be revised. Typical trichostomes, *Plagiopyla*, Colpodidae, appear as prostomial ciliates deformed by allometric growth of the dorsal and ventral kineties (56, 144, 145).

Guilcher (79, 80) has shown that the enigmatic Chonotrichida are related to the hypostomial gymnostomes, the morphogenesis of the bud and the transformation of its ciliature indicating that the peristome of a *Chilodochona* corresponds to the ventral face of a *Dysteria* or a *Trochilioides* in which the antero-posterior axis has been displaced by fixation (64). In the case of the Suctoria, the ciliary patterns of the migratory buds serve as evidence of the unity of the group and of its close general relationship with the holotrichous ciliates, although one has not been able to determine the exact relationship [Guilcher (79, 80)].

The new information cited above for the various groups, taken as a whole makes necessary a complete revision of the systematics of the ciliated protozoa, from the viewpoint of their probable evolution (57). This evolution seems to have taken place in a "closed circuit"; it is characterized by an extreme diversification related, principally, to the variations, differentiations, extensions, or regressions of all or part of the ciliary apparatus, and, secondarily, to the remarkable possibilities of cytoplasmic differentiation in the case of the ciliates (63).

MORPHOGENESIS IN THE CILIATES

Binary fission.—In the prostomial gymnostome ciliates (probably primitive forms) the buccal ciliature appears as a differentiation of the anterior extremity of the somatic ciliary rows; in the course of division this differentiation takes place anew for the posterior individual (opisthe) just below the equatorial furrow. In all the other ciliates the buccal ciliature is a more or less differentiated morphological system, newly formed for the opisthe during fission from an anlage, that is to say, from a field of kinetosomes.

In more simple cases the field of neoformation results from the multiplication of kinetosomes at a restricted location along a certain somatic kinety, this kinety being called "stomatogenous" [Chatton & collaborators; cf. (53, 108, 110)]. But in the heterotrichs (146) and the ophryoglenids (115) a number (often high) of somatic kineties participate in this neoformation. In other cases, the stomatogenous kinety or kineties is or are incorporated in the buccal ciliature which thus becomes an autonomous system, apparently self-reproducible. This autonomization of the ciliary buccal system has been

observed in several orthogenetic series: Lembidae (115), *Paramecium-Frontonia-Stokesia* (39, 55), as well as, with some differences in details, in the Hypotrichina (25), the Oligotrichina (Fauré-Fremiet, unpublished), the Thigmotrichina and the Vorticellidae.

Morphogenesis is brought about during the course of binary fission by a double process: organization of the anlage and the modeling of the new buccal ciliature, and morphogenetic movements assuring proper localization of the buccal ciliary apparatus, such translocation often being realized by allometric growth of several somatic kineties. In the hypostomial gymnostomes with their reduced somatic ciliature a kind of regeneration of some normally absent somatic kineties precedes stomatogenesis (60).

In some instances the somatic ciliature is altered or newly formed during fission. For the gemmiparous ciliates (chonotrichs and suctorians), Guilcher (79, 80) has described new examples of organization of the ciliature by regrouping and multiplication of erratic kinetosomes. When the budding is internal, she has observed first of all an invagination of a group of superficial kinetosomes, and in *Discophrya* proper localization of certain kinetosomes is assured by a curious process of devagination. In the case of the hypotrichs the highly differentiated ventral ciliature is entirely reconstituted, for the proter as well as the opisthe, beginning with the kinetosomal fields whose origins have been described by Chatton & Séguéla (25). For a parasitic ophryoglenid such as *Ichthyophthirius*, the great variations undergone by the surface of the body during growth of the trophont, as well as during the palintomic divisions, are accompanied by a regulation upward or downward, respectively, in the number of kineties [Mugard (116)].

Finally, let us note that in the case of highly evolved forms the development of ciliary anlagen can call to mind, if not recapitulate, certain stages in their phylogenesis (79, 80). *Cyrtocaryum halosidnae*, an astome ciliate similar to *Anoplophrya* in the trophont stage, shows during palintomy a structure typical of an apostome (67).

Regeneration.—Regeneration brings into play the same morphogenetic processes as fission (7, 53, 141, 142). The recent experimental researches performed by Weisz (149 to 162) on *Stentor coeruleus* and *Blepharisma undulans* have contributed important data in this regard. The role of the macronucleus, with the antero-posterior differentiation of its nodes, is shown to be dependent, apparently, upon the degree of polymerization of DNA [or else upon its relationship with certain proteins? cf. (1)]. Furthermore, in *Stentor* the role of the "left boundary stripe" (Weisz) of Schuberg's triangle has been analyzed; kinety number 1 (director meridian of von Gelei) has turned out to be localized there; excision of portions at different levels has shown that it possesses a stomatogenous potentiality throughout its length but actually manifests it only at its anterior extremity. Its presence inhibits the potential stomatogenous capacity of the neighboring kineties which reveal such power only after its total excision. By ingenious experiments Weisz has demonstrated the polarization of the ciliary rows postulated by Chatton & Lwoff [cf. (110)].

On the other hand, in the case of autonomous ciliary systems (cytostome of *Paramecium*, attachment disc of *Licnophora*), Tartar (142), and Balamuth (7) have shown that regeneration cannot be effected by the remaining somatic ciliature.

Bi- or multicomposite systems.—The formation of double individuals can be provoked experimentally either by arrest of fission (49, 52, 53) or by grafting one individual onto another (160, 162). The heteropolar double systems are incapable of giving two homothetic ensembles by division, and they increase themselves by forming multipolar monsters before degenerating. Homopolar doublets alone are viable and multiply as such by division. Their apparent stability is related to the symmetry of the system which expresses itself by an *effet de contrainte;* when this disappears, the processes of regulation promptly reduce the doublets by various mechanisms to the simple, normal form (52).

Other aspects of morphogenesis are due to the development of structures (e.g., the peduncle of the vorticellids) involving secreted parts; new forms can be produced by allometric growth (50). In colonial forms the mode of division of the zooids determines the form of the colony; the case of *Carchesium limneticum* calls to mind several characteristics of the determinate cleavage of the eggs of *Spiralia* (51).

Polymorphism in ciliates.—Several carnivorous ciliates, e.g., *Monodinium vorax* and *Enchelys mutans*, and almost all of the histophagous hymanostomes, show a polymorphism related to their physiological life cycle (47, 48, 65, 115). The succession of stages, theront→trophont→tomont→tomites, characterized more by changes of form than of structure, makes them comparable with parasitic species, e.g., the apostomes, in which the same stages succeed one another accompanied by important modifications of the ciliature [cf., (110)].

All the sedentary forms show, with the formation of migratory individuals, a dimorphism more or less accentuated. In the gemmiparous ciliates (chonotrichs and suctorians) the ciliature of the migrant is newly formed (79, 80); in the sessile peritrichs the development of the ciliated girdle used for locomotion by the migratory form has been studied by Pastori (117, 118) and Ballerini (8). In the folliculinids total regression of the peristome precedes fission; in the course of division the anlage of the adoral fringe evolves differently in the case of the proter and the opisthe (53), giving a normal sedentary individual and an astomatous, vermiform migrant [Andrews (2, 3), Hadzi (81), Hamilton (83)]; after fixation and secretion of its lorica, the migrant resorbs its residual peristome and a new anlage develops into an adoral fringe of normal type.

Among the holotrichs, *Tetrahymena patula* and *T. vorax*, like *Espejoia mucicola*, appear in two different forms, macrostome and microstome; the transformation of the first into the second occurs, after regression of the buccal ciliature, by development of an anlage formed by the stomatogenous kinety [Corliss (34), Fauré-Fremiet & Mugard (66)]. Lithium, which by its "vegetalizing" action inhibits development and ciliary differentiation in

larval echinoderms, prevents appearance of the macrostome form in *T. patula* (54).

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ACID-FAST BACTERIA[1]

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Introduction

Much too late, that is to say long after he agreed to write this article, the present author found that trying to review comprehensively the subject of "acid-fast bacteria" within the allotted space is a hopeless undertaking because an endless variety of recent publications would have to be considered under this heading. It became obvious that a choice had to be made and that in many instances this choice would be arbitrary, leaving out important papers. We wish to make it clear that in most cases the omissions are dictated by this painful necessity and not by willful disregard.

Some subjects are entirely omitted from this chapter. No mention is made of studies related to taxonomic aspects of mycobacteria. The rapidly increasing use of acid-fast bacilli in adjuvant mixtures and their influence on the formation of heterologous antibodies has not been considered. Clinical aspects of the bacteriology of tuberculosis are not reviewed and the entire field of BCG[2] vaccination is left out. Likewise, the use of hemagglutination and other serological techniques as diagnostic tools in tuberculosis is not mentioned. All of these subjects were recently reviewed (1 to 6). Moreover, these fields are so wide and the progress in them has been so rapid that they may need special consideration in a later issue. Of the chemical and biochemical papers, those which have a bearing on pathophysiological problems of tuberculosis and other diseases caused by acid-fast bacteria are primarily discussed. This applies also to studies on chemotherapeutic and antibiotic agents of which only very few aspects are mentioned. Papers reporting mere confirmations or slight variations of previous studies are not, as a rule, enumerated. The present article concentrates on the parasitic mycobacteria and on studies related to their pathogenicity.

The term "acid-fast bacteria" as it is commonly used designates microorganisms belonging to the genus *Mycobacterium* (7). They are characterized by their tinctorial property of retaining carbol-fuchsin or analogous stains after treatment with acid-alcohol or alcohol. The group includes a great variety of saprophytes and a few pathogens: *M. tuberculosis* var. *hominis*, *M. tuberculosis* var. *bovis*, *M. avium*, *M. leprae*, *M. lepraemurium* and *M. paratuberculosis* (*M. johnei*). It is not clearly established whether all mycobacteria originally isolated from poikilotherms (*M. ranae*, *M. marinum*, *M. piscium*, et al.) are pathogenic for the respective animals.

A new type of *Mycobacterium* (*M. ulcerans*) was described in 1948 by

[1] The survey of the literature pertaining to this article was concluded in January, 1953.

MacCallum *et al.* (8). It was isolated from chronic ulcers of the skin in man and is characterized by an optimum growth temperature *in vitro* of 33°C. At room temperature or at 37°C., the organisms fail to multiply. A number of cases have since been reported where a similar organism was isolated from skin lesions, most of them in Africa and Australia (9 to 12). The immunological properties of this mycobacterium were studied by Fenner & Leach (13) and Fenner (14). Despite the cross reactions it gives with other parasitic and saprophytic mycobacterial strains, its immunologic distinctiveness is shown by its reactions *in vitro* as well as by skin tests performed in sensitized guinea pigs.

A similar, new type of *Mycobacterium* which is likewise characterized by growing best at low temperature (31°C.) has been isolated and described by Nordén & Linell (14a). This strain which is probably not identical with the *M. ulcerans* is only slightly pathogenic for laboratory animals.

Three varieties of *M. johnei* were described by Taylor (15) who also devised a method for primary isolation of these organisms (16). A modified medium serving the same purpose is further suggested by Glover (17).

Successful efforts have been made in recent years to classify the acid-fast saprophytes and to describe their cultural and morphological properties (18 to 27). These studies should facilitate the reference to certain strains and the identification of newly isolated ones. Besides saprophytes and the above-mentioned pathogens, a number of attenuated or avirulent mutants or variants are often mentioned in the literature. The most frequently encountered avirulent variants are the H37Ra and the R1Ra strains (28, 29). By far the commonest strain used in the production of vaccine is BCG[2]. Most laboratories preparing BCG vaccine obtained derivatives from Calmette's original strain which is propagated at the Pasteur Institute in Paris, but there are now considerable morphological and cultural differences between the various subcultures (30). Occasionally, the "Vole bacillus" (31) is used instead of BCG for human vaccination.

Following Dubos' nomenclature, we designate as "avirulent" those bacterial strains which do not multiply or cause progressive disease in any animal species, and as "attenuated" those cultures which have a limited ability to multiply in a host, but do not cause a progressive disease under normal conditions. The distinction between virulent and attenuated strains appears to be a quantitative one, whereas avirulent strains are qualitatively different from the others. With respect to their metabolism, however, avirulent strains have maintained many of the original properties of the virulent cultures from which they stem and are clearly different, biochemically, from saprophytes. Virulent, attenuated, and avirulent strains can easily be distinguished morphologically (32).

Various saprophytic strains of mycobacteria have been shown to be susceptible to the action of specific bacteriophages isolated from soil (33 to 38). So far, no phage has been found attacking pathogenic or attenuated strains.

MORPHOLOGY

The morphology of acid-fast bacteria has been studied with the aid of the electron microscope and with other techniques. Werner (39) reviewed the field up to 1951. More recent papers have been published by Knaysi (40), Winterscheid & Mudd (41), Mudd *et al.* (42), and Ruska *et al.* (43). There is general agreement on the presence, in the bacterial cell, of two types of granular bodies: polar inclusions, showing bright refraction of visible light and appearing as dark opaque zones in the phase-contrast and the electron microscope, and more diffuse central areas in which the cytoplasm appears to be denser. There has been considerable controversy concerning the nature of the polar granules. Knaysi (40) maintains his earlier view (44) that they are nuclei, but Mudd and his co-workers (41, 42) and Werner (39, 45) are critical of this assumption. They believe the nucleus to be located in the center area of the cell and to be different in nature from the polar bodies. Werner termed these latter "metachromatic granules." Very recently, Winterscheid & Mudd (41) produced evidence permitting their identification with mitochondria. This conclusion was reached on the basis of the morphological as well as the tinctorial and biochemical properties of the granules. As for the central area, the evidence that it represents the cellular nucleus is not quite as convincing, although there seems to be little doubt that this is the case.

Werner (39, 45) has repeatedly stated that the metachromatic granules (or mitochondria) tend to disappear in aging cells and that they are ordinarily less abundant in organisms of the H37Ra strain than in bacilli from young cultures of H37Rv and other virulent strains. This is an interesting observation which, if followed, could shed some light on the biological functions of these bodies, and perhaps add to our knowledge of the differences between virulent and avirulent strains.

Some of the differences of opinion may stem from the fact that observations made with such different techniques as electron, daylight, ultraviolet and phase-contrast microscopy are often compared. One also has to be aware of the possibility of artifacts disturbing the final picture (46).

Brieger & Glauert (47) studied the multiplication of avian tubercle bacilli. Using the phase-contrast microscope, they distinguish three main types of bacterial multiplication: rods as small as 0.2 μ are released from disintegrating mycelia and grow out to new adult forms, sprouts growing out to filamentous nests and forming spherical colonies, and filaments orienting themselves in "cords." Juvenile forms were often found to be non-acid-fast. With the aid of the electron microscope, Werner (39) observed similar modes of multiplication, especially microrods and sprouts in human-type tubercle bacilli. Using a simple technique of direct agar microscopy, Engbaek & Ørskov (48, 49) confirmed the earlier findings of Middlebrook *et al.* (32) on the characteristics of growth and colony formation of virulent and non-virulent strains of mycobacteria.

CHEMICAL CONSTITUTION

The most recent comprehensive reviews of the chemical constitution of mycobacteria were published by Seibert in 1950 (50) and Lederer in 1951 (51). Reviews were also published by Asselineau & Lederer (52) and Asselineau (53, 54) on lipids and by Seibert (55) on the chemistry of tuberculin. The monograph by Nègre (56) gives a short summary on the chemistry and biological action of lipids and a detailed account on vaccination studies with the so-called "methyl antigen" (*antigène methylé*) of tubercle bacilli, an extract isolated by Nègre & Boquet in 1922 and used for animal as well as human vaccination. In view of the great number of recent reviews, little will be said here about chemical constituents. The discussion is limited to those more recent studies which appear to have a direct bearing on certain biological functions of the bacillus.

Proteins.—Studies on proteins of the tubercle bacillus have been mostly concerned with the isolation of purified constituents having the immunological properties of tuberculin. A number of different compounds were isolated in the course of this work which was reviewed by Seibert in 1950 (50). Following Seibert's suggestion, it is well to distinguish between proteins isolated from heat-killed cultures of mycobacteria or from their filtrates, and constituents obtained from living cultures. Tuberculin and its purified derivatives (PPD and PPD-S) belong to the first of the two groups. For practical purposes, Seibert's PPD and PPD-S are widely used and satisfactory. Their activity has often been compared with that of Old Tuberculin. The correlation is close although not always complete and occasionally OT[2] gives a higher percentage of positive reactions. In an attempt to get a purified preparation, the action of which would be as close as possible to that of OT, Birkhaug *et al.* (57) prepared a "Purified Tuberculin" which was compared in human individuals with OT as well as PPD (57, 58). PT[2] is chemically less pure than PPD but produced a significantly higher number of positive reactions than PPD. Corresponding doses were compared on the basis of their nitrogen contents. PT seems to have the same specificity as OT while still possessing most of the practical advantages of PPD. Further studies will show whether the ratio between specific reactions to PPD on one hand, and to PT and OT on the other, remains the same in larger groups.

The proteins isolated from bacteria and from unheated filtrates of living cultures lend themselves to the study of the activities of the organisms in the host. It may be recalled that three different protein fractions, A, B, and C, have been described (50). These are characterized by their electrophoretic mobilities and by the pH levels at which they precipitate from concentrated culture filtrates. The A, B, and C fractions differ chemically and biologically (50). They all elicit tuberculin-type reactions in sensitized individuals and their potencies can be compared on the basis of their nitrogen contents. This permits the determination of an accurate threshold dose for each preparation. The three products can then be arranged in a potency scale, but it

was found that the sequence in which they range in such a scale is dependent on the way human individuals or animals were sensitized [Seibert et al. (59)]. The threshold dose for each of the products varies according to whether sensitization is caused by BCG vaccination or by infection with virulent strains. This, in a way, is not too surprising since we know of many chemical differences between various bacterial strains as well as between the three protein fractions that were compared. Precipitin and complement fixation tests revealed similar differences between the three preparations.

There has been much controversy in the past on the question of the antigenicity of tuberculin. The fact that intracutaneous tuberculin injections do not convert tuberculin-negative individuals into positive reactors has often been regarded as evidence of the nonantigenicity of tuberculin, but there are sufficient observations to show that in this form the statement is incorrect. Tuberculin-type sensitivity cannot be produced by tuberculin or by tuberculoprotein alone. Only in conjunction with waxes, viz., the lipopolysaccharide fraction of the purified wax of the tubercle bacillus, does tuberculin cause typical delayed tuberculin-type skin sensitivity. This was demonstrated in animals (60) as well as in humans (61). While injections of tuberculin or tuberculoprotein alone do not cause the typical tuberculin-type of sensitivity, these compounds are nevertheless antigenic and produce antibodies which are demonstrable by the classical immunological techniques of complement-fixation and precipitation (59). Finally, tuberculin can desensitize allergic animals or humans which is further proof of its antigenicity [for a review, see Rich (62)]. The failure of tuberculin to induce tuberculin-type sensitivity is connected with the nature of the process of sensitization rather than with the immunological properties of the antigen. Sensitization does not seem to occur in the absence of a cellular reaction about the antigen which is induced only by tubercle bacilli or the above-mentioned bacterial wax fraction.

A mild method of extraction which is suitable for the isolation of various water-soluble proteins was devised by Heckly & Watson (63). It is important to keep in mind that new extraction methods often lead to interesting results. This was demonstrated by the work of Seibert & Fabrizio (64) who adopted a urea extraction method which Stacey and his associates had used for the isolation of polysaccharides (65) and obtained a heretofore unknown protein fraction from live tubercle bacilli which has remarkable antigenic properties. In rabbits, it produces high titers of relatively specific serum precipitins and elicits severe Arthus reactions. Guinea pigs become sensitized to this protein after multiple intradermal injections. This was demonstrated in tissue cultures when exudate leukocytes obtained from sensitized animals were exposed to the specific antigen. The result of the contact was cellular damage and inhibition of the migration of the leukocytes. Since normal exudate cells were not affected by the protein, the antigenicity of the fraction is clearly demonstrated. It appears questionable, however, whether the specificity of the antigen can be determined unequivocally by this in vitro technique.

Other protein fractions (A, B, and C) have similar effects on cells isolated from tuberculous animals (66). Moreover, it should be recalled that inhibition of leukocyte migration is not specific for tubercle bacilli or fractions thereof. Other bacterial products have similar effects (67).

Chemically, the new bacterial fraction seems to be closely related to, although different from the A and C proteins obtained from culture filtrates. The fraction was obtained from both virulent (H37Rv) and avirulent (H37Ra) cultures. From the chemical data published, there appears to be little difference between the products from these two bacterial sources. Most biological experiments, however, were done with the protein obtained from the virulent strain. The new fraction does not in any way protect guinea pigs against subsequent challenge infections. In this respect, it is not different from the A and C proteins obtained from culture filtrates [Seibert (68)].

There are some indications in Seibert's work of a stimulation by tuberculin fractions of antibodies produced by BCG. Such antibody stimulation was further demonstrated by Boyden & Suter (69) who showed that hemagglutinins appear in the serum of BCG-vaccinated guinea pigs four to five days after, but not before the animals were tuberculin-tested. There was no quantitative relationship between the titer of this antibody and the degree of tuberculin-sensitivity, although tuberculin does not stimulate its appearance before the animals have developed some degree of skin sensitivity. A similar though weaker antibody stimulation was also observed in BCG-vaccinated guinea pigs after injections of ovalbumin. Moreover, antibody formation against ovalbumin was stimulated by simultaneous injections of tuberculin. These observations render the specificity of this interaction somewhat doubtful.

The amino acid contents of A and C proteins were analyzed by Seibert & Kent (70). More recently, Pauletta & Defranceschi (71) made a similar analysis of the amino acids present in bacterial extracts of virulent and avirulent strains of *M. tuberculosis*. The differences found were quantitative rather than qualitative. It is interesting to note that they detected the same α,ϵ-diamino-pimelic acid which had previously been found in *Corynebacterium diphtheriae* (72), in a hydrolysate of the lipopolysaccharide fraction of a virulent strain of tubercle bacilli (73) and in hydrolysates of various human, bovine, avian and BCG strains (74, 75). It was not found in extracts of H37Ra (52). Coproporphyrin III was isolated in crystalline form from an alcoholic extract of tubercle bacilli (76).

Lipids.—Excellent reviews on tuberculolipids have recently been published (51 to 54, 77). By continuing and extending Anderson's original work, Lederer, Asselineau, and their collaborators have added considerably to our knowledge of lipid fractions of mycobacteria. In view of the fact that the two most recent of the aforementioned reviews (54, 77) are almost completely up to date as of this writing, the present reviewer will not try to summarize the vast subject in a few short paragraphs, but will limit the discussion to one subject on which some work has been published during the past months.

In 1950, Bloch (78) described a lipid component of the tubercle bacillus which was isolated by superficial petroleum ether extraction from living, young bacterial cultures. It was termed "cord factor" because by this method of extraction it was obtained only from those bacterial strains that formed serpentine "cords" in their colonies, i.e., virulent and attenuated strains (32). "Cord factor" is highly toxic for mice. In subsequent studies, this toxicity was used as an index throughout the work of chemical purification of the compound. It was first shown that petroleum-ether extracts are mixtures of toxic and nontoxic components and that the latter represent but a small proportion of the total extract (79). The toxic component was then separated by chromatography and obtained in concentrated, but not yet pure form (80). The question arose as to whether similar or identical toxic components could not be found in bacterial fractions other than petroleum ether extracts. By using the mouse toxicity test, a comparable component was found in the wax fractions of bacterial extracts from virulent and BCG strains (81). It was finally located in the purified wax fraction of the bacillus and identified as a nonacidic substance different from the known constituents of purified wax (82). Its molecular weight is about 10% higher than that of mycolic acid. It is clearly distinguishable from the latter by its characteristic infrared absorption spectrum and its lack of acidity. In this study, as in other recent ones (83 to 86), infrared spectroscopy was for the first time successfully employed for analyzing isolated bacterial fractions.

Tubercle bacilli, as well as other mycobacteria (87), remain viable after extraction with petroleum ether, which indicates that the toxic material is removed from the outer bacterial surface. Relatively larger amounts are extracted from younger cultures than from older bacterial growths. The accumulation of toxic material at the bacterial surface is inhibited by the presence in the culture medium of small amounts of thiosemicarbazone compounds (TB I)[2] which seem to interfere with the synthesis of the material, or by large amounts of Tween 80 which appear to dissolve the toxic material as it is being produced since, in the latter case, it can be recovered from culture filtrates (88). These observations suggest that the toxic component is a secretory product of the bacteria and that in a lipophilic environment it is released into the surrounding medium. The significant differences in virulence found between bacteria from older and younger cultures of the same strain (89, 90), as well as between organisms grown in the presence and absence of high amounts of Tween 80 in the medium (88), seemed to be suggestive of a definite part played by the toxic component in the pathogenic function of the tubercle bacillus. Some of the experiments dealing with the toxicity of "cord factor" could not be confirmed by Philpot & Wells (91). The reasons for this discrepancy are not easily understandable from their data. Some possible reasons for the divergent results are discussed by Bloch *et al.* (80).

[2] The following abbreviations are used in this chapter: BCG (Bacillus Calmette-Guérin); OT (Old Tuberculin); PT (Purified Tuberculin); TB I (thiosemicarbazone compound).

In continuation of their work on the mycolic acids of mycobacteria, Lederer and his associates described the chemical properties of acids isolated from BCG (92) and from *M. phlei* (93).

Since the early days of tuberculosis research, great efforts have been made toward a better understanding of the physiopathological role of the tuberculolipids. Despite this fact our knowledge is still very dim. It is important to keep the possibility in mind that lipids extracted from bacteria grown *in vitro* may not have the same constituents as are produced by bacteria in an infected host. Observations like the one of Sheenan & Whitwell (94) strongly support the likelihood of this surmise. These authors described the stainability of tubercle bacilli with Sudan Black B. However, only bacteria in smears of sputum or infected organs took the stain, whereas bacilli grown *in vitro* could not be stained by this method.

Polysaccharides.—The most recent survey on this subject by Lederer (51) reviews our present knowledge comprehensively and is up to date as of this writing. The serology of polysaccharides from the tubercle bacillus was reviewed by Iland (95). In his own experiments with seven highly purified polysaccharides, Iland found that they gave precipitin reactions with sera from sensitized animals, but no reactions were found with human sera from tuberculous patients. In view of the voluminous literature (which is not reviewed here) on the hemagglutination reaction with patients' sera using red cells sensitized with polysaccharides, results like Iland's further emphasize the need of using pure antigens in serological reactions.

METABOLISM

Although little time has elapsed since the publication of several excellent reviews on the subject of the metabolism of mycobacteria (96, 97, 98), a large number of papers has accumulated. However, only a brief review of this subject will be made in the present paper.

Of the saprophytic mycobacteria, *M. phlei* has been studied most extensively. Pratt (99, 100) determined the growth requirements of this species and described a simple medium for rapid growth. It was shown that Tween 80 or long-chain fatty acids enhanced the growth rate. Ammonium ions as well as L-glutamic acid can serve as the main nitrogen source, but both have to be present to allow for good growth. The usual amino acid mixture can be replaced by measured amounts of L-arginine, L-histidine and L-proline.

The intermediate metabolism of *M. phlei* was studied by Blakeley (101) who found that bacterial cell debris and dialyzed extracts form citrate from oxaloacetate and acetylphosphate. The author considered this evidence suggestive of the occurrence of a Krebs cycle in this acid-fast microorganism. Turian & Haxo (102) found unknown polyenes, possibly precursors of carotene, in culture filtrates of *M. phlei*, and vitamin B_{12} was isolated from various mycobacterial strains (103). Hanks (104) applied the tetrazolium salt method to measuring the hydrogen transfer capacity of *M. phlei* and of

M. lepraemurium (see below). The same method was also used to measure the hydrogen transfer capacity of tubercle bacilli (104a).

It is tempting to follow Hanks & Gray (105) in their effort to classify the mycobacteria according to their metabolic properties. In a scale extending from the various saprophytes through the tubercle bacillus to *M. johnei*, *M. lepraemurium*, and *M. leprae*, the organisms gradually lose their ability to grow in artificial culture media, becoming less and less able to utilize various common substrates. As their growth rate diminishes, they finally become obligatory intracellular parasites. Such a scheme suggests the existence of metabolic characteristics associated with the pathogenicity of these microorganisms.

A number of investigators have sought to demonstrate metabolic differences between virulent and avirulent mycobacteria and to correlate such findings with the nature of pathogenicity of these organisms. The results, as a whole, are disappointing. In some instances, certain biochemical properties were found to be present fairly consistently in virulent and attenuated strains, and absent in avirulent strains and saprophytes, or vice versa. These cases are briefly summarized in Table I.

In other studies, however, where significant variations were found from strain to strain with respect to a given biochemical reaction, the dividing line that separated the strains according to this reaction followed an entirely

TABLE I

COMPARATIVE TABULATION OF SOME METABOLIC CHARACTERISTICS OF VIRULENT AND AVIRULENT STRAINS OF MYCOBACTERIA

Virulent and attenuated strains	Metabolic characteristics investigated	Avirulent strains and saprophytes	Metabolic characteristics investigated	Ref. No.
H37Rv	Growth retarded by DL-serine, β-alanine, DL-phenylalanine, L-tyrosine.	H37Ra	Growth not retarded by DL-serine, β-alanine, DL-phenyl-alanine, L-tyrosine.	
	Growth slightly retarded by nicotinic acid, hypoxanthine.		Growth strongly retarded by nicotinic acid, hypoxanthine.	106
	Growth not affected by DL-α-alanine.		Growth stimulated by DL-α-alanine.	
H37Rv, BCG, freshly isolated human and bovine strains	Fail to reduce methylene blue, phenolindophenol and similar redox dyes.	H37Ra, R1Ra, saprophytes	Reduce methylene blue, phenolindophenol and similar redox dyes.	107 108 109
H37Rv	Stained by neutral red.	H37Ra	Not stained by neutral red.	110
H37Rv and others	Growth not inhibited by surface-active polyoxyethylene ethers.	H37Ra and others	Growth inhibited by surface-active polyoxyethylene ethers.	111 112
H37Rv	Least increase of oxygen consumption in the presence of L(+) lactic acid.	H37Ra, *M. phlei*	Greatest increase of oxygen consumption in the presence of L(+) lactic acid.	113
H37Rv	Growth stimulated by serum and serum fractions.	H37Ra	Growth not stimulated by serum and serum fractions.	114

different course than the line which parted the various strains according to
their virulence. The oxidation of benzoate is quoted as an example of the
confusion that would result from any attempt to correlate this reaction with
the virulence of strains. Following Bernheim's original observation that the
oxygen uptake of certain mycobacteria is enhanced by benzoic acid, and the
work of Stanier on the intermediate metabolism of benzoate in *Pseudomonas*,
the benzoate metabolism was studied in a number of mycobacterial strains.
It was found that BCG, *M. smegmatis* and *M. stercoris* are able to oxidize
benzoate, protocatechuate, catechol and β-ketoadipic acid, whereas H37Rv,
a virulent strain A27, H37Ra and *M. phlei* fail to do so (115, 116). Catechol
is oxidized with intermediary ketone formation by *M. butyricum*, whereas
BCG oxidizes catechol without intermediary ketone formation and *M.
phlei* fails completely to oxidize catechol (117).

The great majority of virulent strains fail to reduce a number of redox
dyes (phenol indophenol, benzenone, indophenol, benzenone indochloro-
phenol and dibromobenzenone indophenol), whereas the same dyes are
readily reduced by saprophytes and avirulent strains, but in one exceptional
case, H37Ra reacted like virulent strains (109, 109a).

Lactic acid, which enhances the oxygen uptake of avirulent strains more
than of virulent tubercle bacilli (113), is but one of a series of organic acids
(of which capric acid was the most active) which have a strong bacterio-
static action on virulent tubercle bacilli (118). This action becomes more
pronounced as the acidity of the medium increases and is further enhanced
in the presence of serum. A somewhat similar dependence of bacteriostatic
activity on the presence of serum albumin was observed with spermine iso-
lated from beef kidney (119). The presence of certain serum preparations
interfered with the antibacterial effect, but addition of bovine albumin re-
stored the original activity.

In a comparative study on the growth rate of H37Ra and H37Rv, Holm-
gren & Youmans (114) found that in a basal medium the generation time of
the two variants is the same (about 23 hr). When serum or certain fractions
thereof are added to the medium the generation time of H37Rv is consider-
ably shortened, whereas the growth rate of H37Ra remains unchanged.
None of a variety of vitamin preparations have any growth promoting effect
on either H37Ra or H37Rv.

From a study of the differences in growth response of H37Rv and H37Ra
to various metabolites, Marshak (106) suggests that the H37Ra strain is a
single gene mutation of H37Rv and that the two strains differ by their
ability to utilize phospholipids. The avirulent variant has lost this ability.
Furthermore, H37Rv can use two pathways for acetylation, whereas
H37Ra retains only one. However, the evidence for these conclusions is only
indirect and limited.

For many reactions, like the stimulation of oxygen uptake by lactic acid
(113) or the decoloration of methylene blue (107, 108), the differences are
quantitative rather than qualitative. One reason for these quantitative dif-

ferences between virulent and nonvirulent strains might be the fact that the cells of avirulent organisms and saprophytes have a greater surface permeability than the bacteria of virulent strains (107), but not all of these observations can be explained on this basis. For the most part, no apparent relationship exists between the demonstrated metabolic characteristics and the nature of pathogenicity of these organisms.

It is one of the serious shortcomings of leprosy research that *M. leprae*, the presumptive causative agent of the disease, cannot as yet be cultured *in vitro*. (The so-called leprosy bacilli figuring in many strain collections are saprophytes, most of them of dubious descent.) The work of Hanks & Gray (105) must, therefore, be considered of fundamental importance because it appears as the first successful metabolic approach to the problem. Working with a pathogenic strain of *M. lepraemurium* propagated in serial passages in rats (120), Hanks used the tetrazolium salt method for measuirng the cellular hydrogen transfer of these organisms. The method is based on the reduction of tetrazolium salts (tetrazolium violet) to formazans and permits quantitative metabolic studies.

There were characteristic quantitative differences between *M. lepraemurium* and *M. phlei*. While both strains have remarkable hydrogen transfer capacity, this ability is more persistent and stable in the pathogen which exhausts its endogenous hydrogen transfer capacity less rapidly than the saprophyte. Based on a comparison of the behavior of the two strains, Hanks (121) concludes that the tetrazolium method lends itself as an *in vitro* test for the viability and metabolic activity of *M. lepraemurium*. In view of the fact that this organism cannot be grown in any of the usual media, the method promises to substitute for the regular bacteriologcial techniques in studying a variety of bacteriological and pathological aspects of murine leprosy. This expectancy has been further substantiated by the work of Gray (87) who applied the Warburg technique to metabolic studies of the same pathogenic microorganism. The author showed that endogenous respiration can be measured easily and followed for periods of several days. In contrast to saprophytes, but like the tubercle bacillus, *M. lepraemurium* is damaged by anerobic storage and stimulated and stabilized by bovine albumin and protamine and by yeast and liver extracts, but none of these compounds enables the bacilli to utilize any of some 50 different substrates. In contrast to the favorable influence of serum albumin, crude serum sharply inhibits the metabolic activity of the murine leprosy bacillus. Surprisingly enough, this adverse effect is exerted even by serum of the most susceptible experimental host, viz., the rat. Two classes of serum constituents are responsible for the injurious effect, the lipoproteins and the mucoproteins (96). The recognition of this unexpected toxic effect of homologous serum and other compounds commonly used in bacteriological and tissue culture techniques is obviously of utmost importance in any practical approach to the problem of culturing leprosy bacilli *in vitro*.

Most of the above-mentioned metabolic experiments were designed to

add to our knowledge of the nature of pathogencity, but subsequent attempts have been made to use one or another biochemical property as a practical laboratory test to distinguish virulent from nonvirulent mycobacteria. Without reviewing this work in detail, it can be said that the test has yet to be found which could safely, in all cases, replace in the diagnostic laboratory the routine combination of test tube and guinea pig inoculation. It should be recognized, however, that the work of the past few years has refocussed the attention of bacteriologists on many different characteristics distinguishing virulent from nonvirulent strains and that, as a result, more cultures are now probably identifiable without recourse to animal inoculations. The animal experiment is limited to relatively few particularly critical cases.

VIRULENCE OF THE TUBERCLE BACILLUS

The term virulence tries to express a dynamic property of microorganisms in terms of host response to infection, i.e., the resultant of most heterogeneous multiple factors. However, the word is as convenient as it is ill-defined, and we continue to use this misnomer despite its obvious insufficiency, mindful of La Rochefoucauld's warning: "C'est une grande folie de vouloir être sage tout seul." In analyzing the virulence of the tubercle bacillus, we try to follow the advice of another great Frenchman, Claude Bernard: "La science doit expliquer toujours le plus obscure et le plus complexe par le plus simple et le plus clair."

The statement that a bacterial strain is virulent is not saying more than that, under the usual conditions, organisms of this strain are capable of causing a specific disease in man or animal. However, the system of host and parasite comprises so many variables that the virulence of a given strain can be measured only when all of the following specifications are clearly stated: (a) manner in which the bacteria were grown; (b) age of the culture; (c) size of the infective dose, i.e., number of living bacteria (or infectious units) or dry weight of the inoculum; (d) route of infection; (e) animal species, subspecies or strain, age and weight of individual animals; (f) manner in which the animals are kept (food, cages); (g) possible coexistence of concomitant infections or other pathological conditions; and (h) criteria which are used to judge the severity of disease.

Only with all conditions strictly standardized can the virulence of two strains be compared, and conclusions can be drawn from such a comparison only when all remaining variables are kept constant. The following observations illustrate the importance of all the above-mentioned points:

(a) The addition of small amounts of Tween 80 as recommended by Dubos & Davis (122) does not appear to influence the virulence of tubercle bacilli. The concentration of this detergent in the medium can be raised up to 1 per cent without resulting in a conspicuous change in pathogenicity, but 2 per cent Tween 80 causes a sudden drop in virulence without otherwise interfering with the viability of the bacilli (88). A similar reduction in virulence

is caused by a concentration of a thiosemicarbazone compound (TB I) at least 50 times smaller than that needed to influence the rate of growth of a culture (88). The changes induced by these procedures are not genetic variations. In these experiments, organisms isolated from infected animals and cultured *in vitro* under the usual conditions maintained all the properties of the original strain including its original virulence. Obviously, the differences in virulence which are observed in these cultures manifest themselves in the very first phase of the infection before the bacteria begin to multiply. Most likely, more bacilli are destroyed at this stage and the effect is comparable to an infection with fewer organisms. These experiments emphasize the great importance of the early phase of infection in experimental tuberculosis. Exposure of cultures to daylight or sunlight which causes a sharp drop in the number of viable bacilli in a suspension of BCG (123, 124, 125) has similar effects on virulent organisms, too, as has been observed many times in the past.

(*b*) Bacterial virulence is greatly dependent on the age of the cultures used for infection experiments. Nègre (56) and Nègre & Bretey (126, 127, 128) have shown that organisms from very young surface cultures are less virulent than bacteria from somewhat older cultures of the same strain. It is an interesting fact that these young cultures are considerably less rich in lipids including purified wax (129), a lipid fraction which was shown to contain the toxic component of "cord factor" (82). When the pathogenicity of bacteria from submerged cultures, where growth is somewhat more rapid and more uniform, is tested, very young (1 to 2 days old) cultures are likewise found to be less virulent than slightly older ones (3 to 5 days old) (89). In growing older, the bacteria lose a great deal of their virulence and after two to three weeks' growth reach a stage of significantly reduced pathogenicity (89, 130). Fully identical conclusions were reached independently by Swedberg (131).

Nègre's experiments on the infectivity and pathogenicity of organisms from young cultures also appear to have clarified a good deal of the troublesome question of the so-called filterable forms of the tubercle bacillus. Almost all experimental results presented as evidence for the existence of such forms are based on experiments where bacterial cultures or organ extracts containing bacteria were passed through bacterial filters. When the filtrate showed any sort of infectivity it was usually taken as evidence for the presence, in the original culture, of a "tuberculosis ultravirus" or some submicroscopic evolutionary forms of a bacterial life cycle. The correctness of these conclusions could neither be proven nor disproven and the question has had to remain *sub judice* until more convincing arguments could be brought forward for or against them (132), since it could never be safely assumed that the filters retained all bacteria. The recent work of Nègre & Bretey (133) appears to do away with at least part of this old controversy. The authors showed that under specified conditions cellular elements from young surface cultures can pass through "Chamberland L$_3$" filters, the type commonly

used by most French authors who claimed to have isolated a "tuberculosis ultravirus." The bacilli that pass through these filters belong to a juvenile non acid-fast type which Nègre and Bretey have previously described. They do not readily multiply *in vitro* and lack the original pathogenicity of the strain, causing merely an irregular, mild, noncharacteristic form of a "filtration disease." Nègre and Bretey's experiments may not be a key to the understanding of all the contradictory result ever described in this field, but they certainly provide a rational basis for explaining a good deal of the existing confusion.

(*c*) The size of the infective dose has long been recognized as a determining factor in virulence. Since the introduction by Dubos and his co-workers of submerged cultures where tubercle bacilli grow in a dispersed manner throughout the medium and the revival of the use of the mouse as an experimental animal in tuberculosis, a number of studies have brought convincing evidence for the decisive influence that the inoculum size has on the course of the disease (88, 130, 131, 134). It seems to be the experience of most authors, however, that there is a critical maximum dose which causes an acute disease and above which a further increase of the infective dose has no longer a similar enhancing effect (131). Youmans & Youmans have expressed the only dissent from this view (135). These authors conclude from their results that there is a straight linear relationship between survival time and the log of the number of injected bacteria. By increasing this number correspondingly, they reached an infective dose which caused the immediate death of all injected animals. From their mathematical analysis and from control experiments with heat-killed bacteria and with streptomycin-treated groups of mice, the Youmans conclude that mice die whenever a critical bacterial concentration is reached in the lungs. The present reviewer feels a little reluctant to accept this interpretation until more details are given concerning the circumstances under which the immediate death occurs. It is not stated in the Youmans' paper how soon after injection the animals die. A histopathological description of the lesions and a more detailed account of the control experiments seem to be needed if such far reaching conclusions are drawn. On the other hand, this writer shares with the Youmans the experience that the close correlation between infective dose and survival time does not hold when the dose is greatly reduced. With extended survival times, the results scatter over a relatively much wider range and the standard error of the average survival time becomes greater. It can be safely assumed that this is an expression of a developing state of immunity acquired during infection. From all available data, it appears that immunity begins to interfere with the effects of infection when the individual survival times become longer than about 30 days. Most quantitative experiments on this subject have been done in mice because of the obvious practical advantages offered by these animals. One will have to await confirmation in other species before drawing generalized conclusions.

The technique used by Lurie *et al.* (136) to determine the number of bac-

terial units in an aerosol and to infect rabbits by the intraalveolar route has yielded a number of interesting results which show that there is a constant relationship between the number of bacterial units in the inhaled air and the number of grossly visible tubercles found in the lungs. At the same time, Lurie's experiments (137) emphasize the determining part played by the host. Conclusions based on the pathogenic effects of a given number of bacteria are valid only when genetically identical animals are used. Ratcliffe (138) has recently shown that the same aerosol technique is applicable not only to rabbits but, with certain modifications, to mice, rats, hamsters, and guinea pigs as well.

(d) As with most other bacteria and viruses, the route of infection is important in tuberculosis. Intravenous and intracerebral injections and the controlled inhalation of bacteria-containing droplets give the most uniform results. Intraperitoneal and subcutaneous injections usually result in a greater spread of the experimental data. With the possible exception of aerosols, bacterial suspensions used for infection experiments usually contain clumps of varying sizes, regardless of how the suspensions were prepared and in what media the organisms were grown. Whether the existence of such clumps influences the course of the infection in one way or another has never been shown. In one experiment performed on a very small number of mice, equal doses of clumped and uniformly dispersed bacteria had identical effects (89).

With the intravenous route of infection, clumps are retained in the pulmonary capillaries, where they are readily disintegrated by leukocytes. The medium in which tubercle bacilli are suspended also influences their pathogenicity. Paraffin oil (139) and egg yolk (140) have an enhancing effect, the mechanism of which is not clear.

(e) The fact that different animal species show differing susceptibilities to various strains and types of bacilli has been common knowledge for many years and need not be repeated here, but the rediscovery of the mouse as an experimental animal in tuberculosis (140 to 145) has activated the interest in studying strain and family differences in susceptibility as a genetic problem (140, 144). This has been done before in guinea pigs (146) and rabbits (137, 147, 148); however, analogous studies in mice offer the technical advantage of comparing much larger animal series in one single experiment. In view of the considerable standard errors with which one has to reckon, especially in experiments with chronic infections of long duration, the advantages of large experimental series are obvious. The considerable differences in susceptibility between various subspecies, strains and families are important factors to be considered whenever the virulence of two bacterial suspensions is being compared. The results emphasize the predominant role played by hereditary factors in the determination of susceptibility and resistance. An excellent and complete review on native and acquired resistance in tuberculosis was published by Lurie (149).

It appears to be common laboratory experience that the age of experi-

mental animals at the time of infection influences the course taken by the disease and that younger animals are more susceptible than older ones. Swedberg (131) published data showing this to be the case in mice. In guinea pigs, on the other hand, it is claimed that age does not materially affect the susceptibility to tuberculosis (150). It is not clear whether there are sex-linked differences in susceptibility and resistance.

A special sensitivity of the Syrian hamster to BCG was reported by Hauduroy and his co-workers (151, 152). The Syrian hamster (*Mesocricetus auratus*) has long been known to be susceptible to tuberculous infection, but comparative tests did not indicate a higher degree of susceptibility in this species than in the guinea pig. Hauduroy has now shown that hamsters succumb to infection with BCG, dying of a progressive disease which is indistinguishable, histopathologically, from tuberculosis. However, the bacilli isolated from such animals retain their original properties and are not pathogenic for guinea pigs.

(*f*) For a long time, nutrition and housing were considered important factors in human tuberculosis. The understanding is vague, though, and it would be difficult from the available epidemiological data to conclude just what specific factors predispose people to tuberculosis. In housing, it is clearly understandable that crowded squalid conditions favor the spread of airborne and other infections. With respect to nutrition, on the other hand, the concept that susceptibility to tuberculosis is favored by insufficient diets is based on mere impressions, except, of course, in the case of extreme starvation where terminal tuberculosis is often found in people dying from inanition.

The few animal studies that have been made on the influence of diet on susceptibility and resistance to tuberculosis come to the rather unexpected conclusion that higher death rates sometimes occur in animal groups receiving the "better" diets. Dubos & Pierce (153) compared the susceptibilities to severe tuberculous infections of mice fed on four different diets. Considerable differences were found. In their experiments, however, the diet on which the animals were least resistant (85 per cent corn meal, 5 per cent butter, 10 per cent gelatin and salts and in some instances, vitamins and amino acids) was somewhat deficient as judged by the weight curves of non-infected controls. Dubos & Pierce's experiments leave the question open as to whether tuberculosis took a more severe course with the corn meal-butter diet because this regime deprived the animals of a nutrient essential for building up resistance, or whether it supplied a susceptibility-enhancing factor.

A careful study on the same subject was done by Howie & Porter (154). Similarly working with mice, the British authors compared the effects of six different experimental diets on breeding performance and reproduction, the number of young born, the weight and number of young weaned, the number of deaths before weaning, the individual growth rate before and after weaning and, finally, the resistance to tuberculous infection. It is a very in-

teresting fact that the one diet of the six which gave the best results in all breeding and growth experiments was associated with the most rapid progress of tuberculosis. The authors conclude from their experiments "that there is something in the belief of a relation between nutrition and resistance to infection," but their results indicate that the answer might be different from what it is generally believed to be. In a previous similar study, Sengupta & Howie (155) found significant differences in resistance to tuberculosis in two groups of mice which were fed two other diets both of which were adequate and supported growth and reproduction equally well.

These results indicate that diet can significantly influence the susceptibility of an animal species to tuberculosis and that a "good diet" in the sense of growth and reproduction is not necessarily "good" in the sense of conferring better resistance or rendering animals less susceptible to tuberculous infection. Much more information on this interesting subject will be needed, but the results are promising enough to encourage further studies.

(g) Since the only way to measure the pathogenicity of the tubercle bacillus is by the reaction of the host, any deviation of the host organism from its physiological state might influence the course of tuberculosis. This fact is well known to the clinician who fears the combination of tuberculosis with certain pathological conditions, but the mechanism of such additive effects can better be studied in animal experiments. In mice, a concomitant infection of tubercle bacilli with sublethal doses of two penumotropic viruses, a mouse pneumonia virus (PVM) and influenza virus (PR8), had an enhancing effect on the size of tuberculous lung lesions (156). Mild tuberculosis was converted into a more severe disease even when the virus followed the tuberculous infection by as much as 3 weeks.

In a similar way, mild chronic tuberculosis can be converted into an acute fatal disease when cortisone is injected into tuberculous mice and rats (157 to 162). In other species, the effects are much less clear, and it cannot be stated unequivocally whether tuberculous guinea pigs, rabbits, and man react altogether favorably or unfavorably to injections of cortisone (163 to 169).

Not only disturbances of the adrenal hormone equilibrium, but also interference with the normal functioning of other endocrine organs can influence the course of tuberculosis. Lurie and his collaborators (170, 171) showed that estrogen in mature female rabbits had a retarding effect on experimental tuberculosis. It is well to remember that in all instances where normal animals are given hormone injections the doses must actually be regarded as toxic because they do not replace a deficient function. By being given in excess of the physiological demand they grossly unbalance the existing delicate equilibrium and create pathological conditions. To some extent, these experiments can be compared to those of Roche, Cummings & Hudgins (161). These authors found that not only normally resistant rats become susceptible to tuberculosis as the result of large doses of cortisone, but that rats suffering from alloxane diabetes also develop progressive tuberculosis.

The determining influence which a pre-existing pathological condition can have on the susceptibility of the host was most impressively demonstrated by the work of Vorwald *et al.* (172). These authors injected silicotic guinea pigs with the same small doses of BCG as are used for vaccination purposes and found that such animals died from the combined effects of BCG infection and chronic silicosis. The animals died with typical symptoms of progressive tuberculosis, but the bacteria isolated from their lesions still had the original properties of the BCG strain, i.e., they were not pathogenic for nonsilicotic guinea pigs. Neither BCG inoculation nor silicosis alone caused a fatal outcome, but silicosis obviously created a state under which BCG became highly pathogenic for the animals. These experiments can serve as a perfect illustration of the relativity of the term "virulence."

(*h*) Finally, the definition of virulence of a strain depends on the criteria used in measuring and evaluating the pathological changes which the inoculation causes in experimental aniamls. The severity of an infection can be expressed in terms of the time elapsed between the day of inoculation and the death of the animals in a group. The 50 per cent (L_{50}) value usually comes close to the mean survival time. Although it is not quite as informative as the average suvival time, the advantages in terms of time and space savings are so obvious that it is now widely used. Other methods consist in evaluating and rating the extent, distribution, and the histopathological nature of the gross anatomical lesions, the number of viable bacilli they contain, the absolute and relative weights of lungs, liver, spleen, etc. Many systems have been suggested, but it does not seem feasible at the present time to unite all criteria in one generally acceptable master scheme.

It should be clear from this analysis that statements about the virulence of a bacterial strain have to be based on observations which take all of the above-mentioned variables into account. They can therefore by definition be of only relative significance. Comparative studies of the virulence of two or more strains have to be carried out under strictly constant experimental conditions.

The classical example of the different response of rabbits on the one hand and of guinea pigs on the other to infection with human type tubercle bacilli shows that animal species vary not only in their general susceptibilities toward tuberculous infection, but that their relative susceptibilities to different bacterial strains or types can vary so much that virulence tests in one species may completely reverse the order of results obtained in another. Realization of this fact will make it understandable that there must be poor parallelism between the virulence of tubercle bacilli as determined in an animal inoculation experiment and the severity of the clinical condition of a tuberculous patient from whom the tubercle bacilli were originally obtained. This is, in fact, the conclusion reached in most studies of this nature (131).

Interaction between tubercle bacilli and leukocytes.—The influence of tu-

bercle bacilli on host cells has been given special attention by many investigators. Although the tubercle bacillus grows well in a variety of simple culture media, it comes close to being an obligatory intracellular parasite in its natural habitat. To know whether a bacillus growing within a cell has the same properties as organisms propagated in a culture medium and, on the other hand, to understand the effect which a multiplying bacillus exerts on the host cell in which it resides are most important problems. Indeed, they reach to the very core of the entire mechanism of the host-parasite relationship in tuberculosis.

Tissue culture techniques can be used for studying the behavior of cells from normal animals as well as from animals sensitized to tubercle bacilli. Recently, a number of investigators have used leukocytes obtained from buffy coats or from peritoneal exudates. There is good evidence that in the earliest stages of infection tubercle bacilli are engulfed by leukocytes and that in later phases of the disease they are taken up mostly by macrophages. Conclusions drawn from findings of *in vitro* experiments with leukocytes therefore may well have biological significance. No attempt is made to review the entire field of the propagation of tubercle bacilli in tissue cultures and the reactions of the animal tissue under various conditions. Comprehensive articles have been published recently (62, 173, 174, 175).

A number of older observations suggested that phagocytes were injured by phagocytized tubercle bacilli and especially so by multiplying organisms. Similar conclusions were drawn by Bloch (176), who injected virulent (H37Rv) and nonvirulent (H37Ra) bacteria intraperitoneally into mice and followed the quantitative distribution of intra- and extracellular bacilli and the ratio between free and bacteria-containing cells. The findings suggested that both virulent and avirulent strains are phagocytized at the same rate, but that engulfed bacteria of the virulent strain damage the phagocytes to the point where they undergo lysis and release the bacteria again, whereas the avirulent organisms do not have a similar harmful effect on the cells. These observations were made within a few hours after infection and indicated a direct cytotoxic effect not dependent on bacterial multiplication. Similar differences between virulent and avirulent strains were shown to exist with regard to the migrating activity of leukocytes which had engulfed bacteria of virulent or of avirulent strains. The migration of leukocytes containing virulent bacteria is inhibited, while this is not so evident in the case of leukocytes which have engulfed avirulent organisms (177, 178). As mentioned before, this effect is not specific, since the migration of normal leukocytes is also inhibited by larger numbers of avirulent bacteria (179) and by other bacterial products (67).

Suter (180) cultured monocytes containing phagocytized tubercle bacilli in roller tubes. By adding enough streptomycin to the culture medium, extracellular bacterial multiplication was stopped without interference with intracellular bacterial growth. Suter's data show that virulent bacteria

(H37Rv) multiply within guinea pig monocytes whereas avirulent bacteria (H37Ra) do not, that phagocytes containing virulent bacilli are damaged and disintegrate, that attenuated strains (BCG, R1Rv) have a similar though milder effect than do virulent organisms, and that the cytotoxic effect of these as of all other strains is dependent upon the size of the infective dose. It is interesting to note that in these experiments, as in a previous study [Suter & Dubos (30)], Suter found a distinction between two variant strains of BCG ("Phipps" and "Tice"), the "Phipps" strain being more destructive to the cells than the "Tice." It is consistent with the views expressed elsewhere (32, 78) that of the two strains, the "Tice," which morphologically, i.e., with regard to its ability to form cords, resembles the avirulent H37Ra strain, should be less injurious to the host than the "Phipps" strain which in its appearance is closer to the virulent strain H37Rv (30). Good agreement between the pattern of growth and virulence of various strains was also reported by Yegian & Kurung (181).

Using a somewhat different technique, Mackaness (182) also found virulent tubercle bacilli acutely toxic for monocytes, causing cellular lysis and disintegration due to toxicity and not to mechanical disruption. Bacteria of the H37Ra strain did not multiply and their presence did not injure the cell. These findings match Bloch's and Suters' very closely. There seems to be no doubt that virulent and attenuated bacteria multiply intracellularly, that their presence and multiplication within leukocytes is injurious to the cells, and that in these respects they are basically different from avirulent variants of the H37Ra type.

Suter (183) as well as Mackaness (182, 184) applied their techniques to the study of the actions of various drugs on intracellular tubercle bacilli. They found that streptomycin penetrates the cells and affects the bacteria only when present in very high concentration in the extracellular fluid, whereas isoniazid appears to diffuse very readily into the cells and is effective in about the same concentrations that are needed to prevent bacterial growth in an ordinary culture medium.

Drug Action and Drug Resistance

Attempts have been made to explain the action of chemotherapeutic drugs on the basis of specific interference with enzyme systems. Zeller *et al.* (185) found that diamino-oxidase from *M. smegmatis* is inhibited by streptomycin. Diamino-oxidase from streptomycin-sensitive strains of human tubercle bacilli was considerably more sensitive than an enzyme obtained from *M. smegmatis* and from streptomycin-resistant strains of mycobacteria. The authors first suggested that this specificity might explain the mechanism of action of streptomycin. Later, however, they found (186) that a great variety of antibiotic agents and synthetic amines had similar inhibitory actions on diamino-oxidase from mycobacteria as well as other microorganisms. Although there were, among the latter group of substances

chemotherapeutic agents like Sodium pp'-diamino-diphenylsulfone-NN' didextrose sulfonate (promin) and "TB I," the authors now conclude that this enzyme inhibition does not seem specific enough to explain necessarily the action of streptomycin. The isolation from bacterial culture filtrates of a histidine-like metabolite by Smith *et al.* (187) is perhaps interesting in connection with bacterial diamino-oxidase.

Di Fonzo (187a) made an attempt to explain the bacteriostatic effect of *p*-amino-salicylic acid by this drug's interference with the purine catabolism of the bacillus. Various purines are attacked by bacterial enzymes and there were considerable quantitative and qualitative differences among four saprophytic strains, BCG and H37Rv.

After Pope & Smith (188) had found that tubercle bacilli are able to synthesize biotin, Pope (189) tested a number of synthetic biotin antagonists and found that these products are growth inhibitory *in vitro*, but that they are devoid of such effect if tested on infected chorio-allantoic membranes. The *in vitro* inhibition is specifically reversed by biotin.

Streptomycin-resistant and streptomycin-dependent variants of *M. tuberculosis* have been isolated by several authors (190 to 192a) but, as yet-such strains have not been much used for biochemical studies on the mechanism of drug resistance. Peterson & Pope (193) found small, but not necessarily significant metabolic differences between a streptomycin-resistant variant of H37Rv and the parent strain.

Shortly after the introduction of isoniazid as a chemotherapeutic drug, the occurrence of highly resistant strains of tubercle bacilli was reported from numerous laboratories. There is no need to enumerate individual papers. They all come to the same conclusion, that high degrees of drug resistance are rapidly acquired by the tubercle bacillus *in vivo* and *in vitro*, under experimental conditions as well as in the course of clinical treatment, and that nothing is known about the underlying mechanism.

CULTURAL MEDIA AND BACTERIAL GROWTH

New modifications of existing culture media and ot fhe methods of preparing pathological specimens for inoculation have been suggested by many authors. Most of these publications describe a method which in a certain laboratory gave satisfactory results or proved superior to other methods. As can readily be seen from many contradictory statements, this does not necessarily mean that the same modifications are found superior by other workers too, and very often it seems impossible to judge from the literature why such discrepancies exist. Since we do not have sufficient space to describe all these technical papers, we merely refer to recent publications summarizing critically all the techniques used in the diagnostic laboratory. These include a new edition of a book on diagnostic and experimental methods in tuberculosis by Willis & Cummings (194) and a symposium (195, 196, 197) on the same subject by Smith, Cummings, and Weed. A

selection of recent papers pertaining to the same subject is supplied without further comment (198 to 214a).

The plate count method for the enumeration of viable bacterial units was carefully worked out by Fenner *et al.* (215, 216). It seems, however, that most authors prefer the more convenient method of turbidity measurements. A different method is used by the Youmans (200), who measure the generation time of a culture by determining the appearance of the first visible growth after minimum inoculation. A specific mode of bacterial multiplication, "arithmetic linear growth," is described by Fisher *et al.* (217, 218), who found this to be the prevailing type of growth in many mycobacterial strains. It is regularly observed after a relatively short initial period of logarithmic multiplication.

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GENETICS OF MICROORGANISMS[1,2]

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Introduction

The literature on genetics of microorganisms continues to develop at a rapid rate. Of this year's volume of the Cold Spring Harbour Symposium which was entitled *Genes and Mutations* more than half was devoted to microorganisms. Whether the microbiologist will eventually abdicate this field to the geneticist is not evident at the present time. Certainly, as long as contributions are to be made with regard to methodology, species and population concepts, and special applications useful to the applied phase of microbiology we may expect efforts in this field to be shared by both disciplines as is done at present by microbiology and biochemistry with metabolism and nutrition. Consequently we may expect duplication in microbiology and genetics review journals. The Microbial Genetics Bulletin, now in its fourth year, serves admirably as an informal medium of contact between workers in this field. It is possible (although in our judgment this is not imminent or even desirable) that the tools, methodology, and terminology in this area may become sufficiently unique so that a new discipline may evolve as biochemistry developed between chemistry and physiology.

Methods

Several methods of interest to workers in microbial genetics have been introduced or developed during the year. Bryson & Szybalski (25) have reviewed the methods for isolating resistant strains; they have illustrated in greater detail applications of the gradient plate procedure and have presented a new instrument for selecting resistant organisms in a continuous broth culture. This device, the turbidostatic selector, adds new broth and increasing concentrations of inhibitor as dictated by turbidity measurements with "only occasional attention from the operator." An ingenious method for selection in a single transfer of organisms highly resistant to pH sensitive antibiotics was developed by Matney & Mefferd (129). It depends on using a proper medium, an acid-producing organism, and an antibiotic level that inhibits all but a few resistant organisms in the inoculum; when these resistant mutants grow and produce acid the increased activity of the antibiotic inhibits all but the more resistant mutants of the original mutants, and the selective action pyramids without further attention from the

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[1] The survey of literature pertaining to this review was completed in December, 1952.

[2] The following abbreviations are used in this chapter: FA (filterable agent); EMB (eosine methylene blue agar medium).

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operator. Gezon & Collins (72) employed a constant treatment technique to select antibiotic resistant organisms which involved inoculating medium in an inverted hollow porcelain filter candle and placing this in a bottle of broth containing the antibiotic. The antibiotic concentration in the broth outside the filter candle was increased at intervals and good increases in the streptomycin- or aureomycin- but not penicillin-resistance was obtained. Olitzky (146) found the most probable number procedure, as used in water bacteriology, checked with direct plating for determining numbers of drug resistant mutants and observed that the numbers of mutants increased as the *Brucella* cultures aged.

The replica plate method of Lederberg & Lederberg (112) involved stamping with a sterile velvet pad a series of replicates of plates seeded with a definite pattern of colonies to permit more convenient study of large numbers of individual clones. When used on plates with increasing antibiotic concentrations the plates with the highest concentration showing growth indicated from which portion of the control (no antibiotic) plate the resistant organisms could be isolated. By repeating this procedure with plates seeded from the indicated portion of the control plates, cultures were isolated that were resistant to streptomycin although they had never had any contact with the drug. The procedure worked especially well with isoniazide [Bryson & Szybalski (25)] permitting isolation of fully resistant strains without using a drug-containing medium. Lieb (118) has used the replica method to test colonies of lysogenic strains for lysogenesis by printing them on a plate seeded with an indicator strain. A rough estimate of the amount of free phage could be made.

Since mutant *Neurospora* conidia inhibit wild type, Grigg (77) suggested that back mutation experiments may be invalid for estimating mutagenic action. The observed increase in prototrophic clones as a result of mutagenic action may be only the killing of mutant conidia thus permitting the development of more wild type conidia already present in the population. The Davis-Lederberg and Zinder penicillin procedure for isolating biochemical mutants was found useful by Goldstein (74) in enriching for streptomycin-dependent organisms which thus were shown to be present in a normal population in the absence of streptomycin. Whether exposure of a growing population to penicillin permits the survival of the nongrowing streptomycin-dependent organisms, or whether it merely selects for slow growth which is favorable *per se* to mutation to streptomycin-dependence, is not clear. Adelberg & Myers (1) have modified the penicillin procedure to eliminate (*a*) cultivation in liquid medium with the resultant possibility of selection and (*b*) contact with growth factors prior to the plating thereby reducing the manipulations (washing, starving, diluting, etc.) to three successive agar layerings. These layerings, which follow plating of the survivors of irradiation on minimal medium and growth for about 7 hr. to permit colonies to develop to about 100 cells and mutants to divide until auxotrophy is expressed, are (*a*) penicillin agar to inactivate wild type, (*b*) penicillinase

agar, and (c) agar with supplemental nutrients. By using proper intervals between the addition of the layers, and by marking colonies that appear before the last step, one finds that at least 90 per cent of the colonies which require final consideration are auxotrophic mutants. Forbes (63) has developed a procedure which promises to be useful for selecting auxotrophs in filamentous fungi. It employs sulphur dioxide which in proper concentration kills germinating conidia without affecting those not germinating, and its action can be readily neutralized by the addition of nontoxic amounts of permanganate.

Haas *et al.* (79) studied seven histidineless mutants of *Neurospora crassa*, none of which were able to accumulate any product in the medium that was biologically active for the other mutants. Crossing the strains and producing double mutants enabled them to provide evidence for a biosynthetic sequence and to arrange the mutants in the order of occurrence of their genetic blocks. Horowitz & Leupold (89) offered evidence in support of the unifunctional action of genes; by using the temperature-sensitive mutants as a base it could be shown that the known biochemical mutants of *Escherichia coli* and *Neurospora* are not a highly selected sample from which the multifunctional mutants are excluded by the screening procedure. Application of this method reveals that over 75 per cent of the biochemical mutants of *E. coli* and over half of the biochemical mutants of *Neurospora* are recovered by usual screening procedures.

Kann (99) studied sexual recombination of *E. coli* in liquid media and believed that with nonsyntrophic strains the liquid medium method should prove more suitable for the study of the kinetics of the process. Emard & Vaughn (58) have developed a sorbic acid medium which inhibits catalase-positive bacteria and could be useful for enumerating catalase-negative mutants.

Modifications in techniques with phages which should be valuable to the geneticist have been introduced. Benzer (14) used the change in radiation sensitivity as a tool in following the development of phages inside the cell; Luria (121) modified procedures for estimating the frequency of bacteriophage mutants; and Hershey & Chase (86) analyzed the mutations obtained in bacteriophage recombination experiments.

THE ORIGIN OF VARIATION IN MICROORGANISMS

There still exists a controversy concerning the origin of certain types of adaptedness encountered in microbial populations. There is general agreement that organisms are prefitted by their heredity to a normal range of conditions and that adjustments can be made within the fixed limits set by heredity. Weiss (192) pointed out that the only generalization that can be drawn safely is that the latitude left to "on the spot" adaptation is extremely narrow as compared to the wealth of inherited adaptedness of evolutionary origin. The ratio between the two varies from species to species and from function to function. Most microbiologists today support the view that

most phenotypic changes observed in cultures result in the first instance from the occurrence of a spontaneous or induced mutation which produces a strain that is better able to survive and multiply under the existing environmental conditions than was its parent strain. If the environment is changed certain other mutants which would not survive or be apparent in the old conditions (but nevertheless existed) would have a better chance for survival and multiplication in the new surroundings and would yield the secondary parent culture. Usually such changes take time to become observable in the culture. Newcombe (142) has shown in his studies on phenomic lag that mutations known to be induced at a certain time required several generations for the mutant change to become phenotypically visible. Usually the change which is observed is not the direct result of the mutated gene but is the product of an altered enzymatic process which is itself influenced directly or indirectly by the mutated gene. Thus, normally, it will take some time after the mutation has occurred to build up the changed enzyme necessary for the appearance of a new product and to deplete the original enzyme.

The replica plate method of Lederberg & Lederberg (112) has offered strong support to mutation and selection theory since it has been used to demonstrate the occurrence of drug-resistant individuals in a population which had never been exposed to the drug. Nevertheless, the intervention of the environment in some modifications of heritable features in microbial populations, in addition to its role in the selection process, has strong support, especially in the case of drug resistance. However, it is not clear whether the drug produces in living cells "certain configurational changes which would be compatible or incompatible with the specific configuration of the drug" [Sevag & Rosanoff (170)], whether it acts as a mutagenic agent, whether it induces enzymic adaptations which account for the change, whether the adaptation or injury [Voureka (187)] renders the cells more liable to mutation and selection, or whether the adaptation must occur after the mutation accident but before the mutants can grow to mature cultures. It appears reasonable that instances exist where one or more of these processes supplement the mutation and selection process, and there is certainly some adaptation involved in the manifestation of any mutant. Some of the confusion has arisen from the failure to appreciate that adaptive enzymes are under genetic control and that the adaptive ability may be gained or lost by mutation. In this respect there is an analogy to temperature mutants in bacteria and *Neurospora;* only under certain conditions are the characteristics exhibited which permit them to be recognized as mutants. Ryan (164) has shown these relationships clearly in the case of lactose adaptation in *Escherichia coli.* The lactose-positive (*lac+*) strain of this organism has the inherited ability to grow on lactose after being exposed to this substrate for a time sufficient to generate the enzyme lactase. The *lac+* strain was derived by mutation from a *lac−* strain which would not synthesize lactase in the presence of its substrate; in other words, the *lac+* strain possessed a gene necessary for the adaptive formation of lactase. In complete medium,

mutation from *lac —* to *lac+* occurs at random and is independent of the presence of lactose, and so the two strains are indistinguishable; but, when placed on lactose medium the *lac+* mutant has a selective advantage since it can develop lactase and will overgrow the *lac —* parent.

Mundkur (140) made similar observations on yeast and showed that the divergent views concerning long-term adaptation to galactose utilization in yeast arise from strain differences. Two different modes of adaptation to galactose occur. In one, yeast cultures ferment galactose after long and irregular delay by the emergence of a mutant which is able to ferment this sugar rapidly; such mutants are highly stable, can be tested by genetic crossing, and are caused by a specific mutation occurring in the slow background growth of the parent strain. The second type possesses the inherent ability to adapt to galactose fermentation; such cultures regularly achieve adaptation on the sixth or seventh day after contact with the sugar, and then they quickly revert to nonfermenters when contact with it is broken. Stable rapid-fermenter clones may arise from these cultures by mutation and overgrow the adaptive-fermenter when the yeast is grown in the presence of galactose.

The analysis of the lactose adaptation by Ryan (164) should clarify a number of problems where simple mutation and selection do not appear to explain the experimental data. Sevag & Rosanoff (170) reported that contact with streptomycin was necessary for the acquisition of resistance to this antibiotic in *Micrococcus pyogenes* var. *aureus* and they intimated that streptomycin was the inducer. The sensitive cells did not acquire resistance when grown in a glucose-free amino acid medium or in a medium containing glucose but deficient in aspartic acid or phenylalanine; resistant clones were obtained when the sensitive cells were grown in complete amino acid medium containing glucose and streptomycin under aerobic conditions. After acquiring resistance the cells grew in the deficient medium and the resistance was stable. One resistant cell added to a large population of sensitive cells could be recovered from the deficient medium, suggesting that streptomycin blocks the synthesis of phenylalanine and aspartic acid in sensitive cells and consequently blocks the emergence of streptomycin-resistant cells. This blockage is either antagonized or bypassed when these amino acids are present and when resistant cells develop. Both the sensitive and resistant cells are capable of synthesizing these amino acids in the absence of streptomycin, and since no resistant cells are found in any population inoculated into the amino acid deficient medium containing streptomycin, it was concluded that there were no resistant cells at the beginning. However, these results could also be indicative of blockage of formation of an adaptive enzyme in rare mutants which would not be formed unless streptomycin were present together with phenylalanine and aspartic acid. This enzyme is thus in the paradoxical position of requiring streptomycin stimulation for its synthesis, and yet its synthesis is prevented by the interference by streptomycin with the amino acid building-blocks. The effect of a modified

metabolic state of the cell on adaptive enzyme formation was demonstrated by Pinsky & Stokes (150, 151). They showed that the relatively poor adaptability of young cultures in the case of formic hydrogenlyase was a result of unsuccessful competition of the enzyme building system with other synthetic processes in the cell. As the culture developed and the other synthetic processes slowed down, more building blocks become available for enzyme synthesis, and adaptation took place.

Eagle *et al.* (55) reported that with some antibiotics a continuous spectrum of resistance occurred in the bacterial population instead of the distinct steps predicted by a mutation process. Demerec *et al.* (47) pointed out that some countable colonies which appear on antibiotic-containing agar plates are actually partially inhibited by the level of drug used, but they make sufficient background growth to give rise to more resistant mutants (observable microscopically as papillae on the partially inhibited colonies), so that subcultures show the colony to be resistant to considerably more inhibitor than was in the plate on which the colonies were grown. Thus the methodology obscures the observation of the true resistance of the cell planted on the agar plate, especially at low levels of the inhibitor. Taking this into account, it is possible to set up an hypothetical case in which two mutations would account for resistance occurring as a continuous function. If the organism should become more resistant by virtue of increased content of some inhibited enzyme, and if the activity of the drug on the enzyme were governed by the cellular pH, and if each of these could be mutated to a new mean value with a normal distribution among the cells of the same genotype, then all levels of resistance could be present in a population.

Dean & Hinshelwood (41) have investigated colony formation by *Bacterium lactis aerogenes* on solid media containing different antibacterial agents, and they arrive at the conclusions that variances between different cultures grown on the same type of medium show no relation to ease of production of resistance and that, because so many factors influence growth, it is not surprising to find that there may be greater variation between samples from each of a number of cultures than between a number of samples from the same culture. In their experiments differences in scatter were quite as large with phenol, where they state no adaptation occurs, as with chloramphenicol, where good adaptation results. However, Berger & Wyss (15) have found a considerable range of adaptation to phenol under certain conditions, and this seems to be the result of adaptation superimposed on mutation. Furthermore, while variance analyses are not in agreement with adaptation, they are in exact agreement with predictions of random mutation theory, and, in those few cases where the analyses have not supported this, errors in the evaluation of mutants, such as background growth, have been shown to be responsible. In answer to the argument that forward and backward mutation rates should bring about an equilibrium Ryan (164) points out that a bacterial population is not static but that new mutants constantly appear, some of which are fitter types for the environment exist-

ing at that time. Such mutants will overgrow and eliminate the parental type, and then they in turn are eliminated by the same process. Periodic selection occurs frequently enough to prevent any specific mutation from attaining true mutational equilibrium and is responsible for the fact that a "thoroughly trained" strain when mixed with wild type is stable when it is removed from its selective medium, while "poorly trained" strains in the same conditions are not. By periodic selection the thoroughly trained strain has become faster growing, and, while the unadapted bacteria will probably have a selective advantage over adapted bacteria of the same growth type, they certainly would not have an advantage over adapted bacteria of a superior growth type.

Ravin (157) and Ravin & Norman (158) have reported on studies on the adaptation of *Aerobacter aerogenes* to growth on single carbon sources. This organism will show adaptive ability to grow on acetate, succinate, fumarate, and α-ketoglutarate after being grown on glucose, and this adaptation does not affect its ability to utilize glucose. This appears to be a case of true adaptation and not the selection of rare spontaneously occurring mutants. The adjustment process is subject to two kinds of obstruction; one of these is adaptive (and unstable), while the other is genetic (and stable) and is shown to arise by mutation. Ravin points out that previous investigations suggest that the Krebs cycle normally does not function in the aerobic dissimilation of glucose in *E. coli* but that it constitutes a potentially active system within which adaptation might occur. The results of his experiments with a citrate utilizing strain (C+) of *A. aerogenes* and its mutant which is incapable of using citrate (C−) are in agreement with this hypothesis and also show that the ability to produce the adaptive enzyme is under genetic control. The reactions blocked in the C− mutant probably do not occur in the wild type C+ strain when it is growing on glucose. This contention is supported by the fact that on glucose the growth of C− and C+ is the same, and by the fact that the class of compounds which C− cannot utilize is exactly the same as that class to which the C+ strain can adapt after growth on glucose. Thus the reactions leading to adaptation which are set in motion in the wild type cells in citrate media are probably those which are blocked in the mutant. Szulmajster, Grimberg-Manago & Delavier (181) have arrived at a similar conclusion from their studies on adaptation to citrate utilization in *E. coli*.

THE MUTATION PROCESS

It now appears that earlier conclusions on the similarity of spontaneous and induced mutants, which held the induction process to be a nonspecific way of producing more mutants of all types, were based on insufficient evidence. As more loci and more inducing agents are studied there appears to be a considerable variation in the responses obtained, and some loci appear to be affected not at all by any action which is highly mutagenic for another locus. The comparison of spontaneous and induced mutations [Newcombe

(143)] showed that different loci are differentially sensitive to several mutagenic agents. In certain materials multiple mutants occurred more frequently than would be expected on the basis of simultaneous origin of independent mutations in a single individual [Loutit (120)]. Bryson & Davidson (24) suggested that a mutation causing one class of phenotypic change greatly increases the probability of a simultaneous or rapidly ensuing mutation of another type which would occur independently only at a much lower rate. That is to suggest again the introduction of an instability which may affect a class of loci.

Delayed expression of mutants.—Delayed expression of mutants was compared with phage resistance, streptomycin resistance, and reversions from three nutritional deficiencies by Demerec *et al.* (57). However, there is a difference in that following ultraviolet irradiation essentially all mutants to streptomycin resistance appeared by the fourth division, but with phage resistance from ten to twelve divisions were required for full expression. This shows that neither (*a*) a multinucleate condition, (*b*) reduplication of structures, or (*c*) variability in onset of time of division can be held solely accountable for the delay since these would cause the pattern for the delay to be identical for different mutations. The pattern for delayed appearance of mutations is determined by the locus, not by the mutagen. From studies of lactose negative sectors in colonies sprayed with phage it was evident that the multinucleate appearance could not be entirely responsible for sector formation. However, additional evidence indicated that sectoring could be correlated with the number of nuclei in the cells. This, together with the increase in over-all frequency of induced mutants in multinuclear cells, supported the generally accepted but experimentally unsupported assumption that the cytologically demonstrable body in bacteria which is termed the nucleus actually carries the genetic material. However, when irradiated streptomycin-dependent bacteria are plated on EMB[2] containing no streptomycin, the colonies appearing should have no sectors, since the only colonies that appear should be the selected segregants derived from single nuclei which carry induced mutation to streptomycin nondependence. Yet sectored colonies did arise among the double mutants though in reduced numbers.

Radiation effects.—In current investigations on the effects of radiations an attempt has been made to clarify the action of indirect effects of various radiations in bacterial killing, in virus and enzyme inactivation, and in mutation, and to correlate irradiation effects with those of the chemical mutagens. McElroy (125) has investigated the possible occurrence and nature of intermediates formed during mutation to determine if the delayed effects observed with x-ray and ultraviolet light were caused by the formation of actual products which remained in the cytoplasm or were a result of the gradual stabilization of a modified energy state within genetic material. Previous experimental results showed that nitrogen mustard-induced mutations were reversible for some time after the mutagenic agent had been removed by applying pressure during, or immediately after the mutagenic

treatment. These results indicated the formation of a "transitory, semi-activated complex which eventually decomposes either to the original state or to a new mutated state." High pressure applied to a microconidial strain of *Neurospora crassa* after the irradiation led to either no change, an increase in the mutation rate, or a decrease in the mutation rate, depending on the ultraviolet dose. If the dose were low enough so that more than 5 per cent of the microconidia survived, then pressure decreased the percentage of delayed mutations, while if survival was less than 5 per cent the pressure increased the delayed mutation rate. Further experiments to determine if pressure would have a comparable differential effect on high and low concentrations of nitrogen mustard and how the two act in combination with ultraviolet light led McElroy to believe that nitrogen mustard combines in some way with certain chromosomal or genetic constituents and that this reaction is not pressure-sensitive. This combination, however, alters the chromosome or gene to render it sensitive to supplementary mutagenic agents. When the activated genetic material is acted on by a secondary mutagenic agent it increases in volume (the pressure sensitive step) and then decomposes either to the original state or to a new mutated state. If a semistable intermediate is formed during the after-treatment with a mutagenic agent, its decomposition probably would be affected by temperature. Experiments with both *Neurospora* and *Aspergillus* suggested that the delayed effects of ultraviolet irradiation were not caused by the production of a chemical mutagen which then can remain in the cytoplasm for a considerable period before acting, but rather by the formation of "semistable intermediates" in the mutation process which require time for stabilization.

Swanson (180) found supplementary agents which are not effective mutation agents when used alone, but which when combined with x-ray or ultraviolet treatment increase or decrease the frequency of chromosomal changes (deletions, breaks, etc.) without affecting the induction of mutation and *vice versa*. The mutations induced by a given dose of ionizing or photochemical radiation may be modified by supplementary agents such as infra-red, temperature changes, or dilute nitrogen mustard. These supplementary treatments may affect the rate of survival of ultraviolet-induced mutations, and this is detectable only at ultraviolet doses higher than those which determine the peak of the mutation curve. The x-ray- and ultraviolet-induced mutation rates are not affected in the same way since mustard pretreatment does not affect the x-ray-induced mutation rate but does affect that induced by ultraviolet. Supplementary treatments which modify the rate of chromosome rearrangements may or may not affect the mutation rate, since it has been shown in *Tradescantia* and *Drosophila* that pretreatment with infrared radiation increases the frequency of chromosome rearrangements but does not necessarily increase the mutation rate; the frequency of mutations obtained when x-ray and ultraviolet are used in sequence is much higher than would be expected if the two types of radiations act independently, emphasizing that the process of mutation-induction is

an indirect one proceeding through intermediate stages which may be affected by such supplementary agents.

Kaplan (101) has compared the induction of several mutations in *Serratia* by ultraviolet of different wavelengths. A mathematical treatment of mutation to different colony colors and colony sectoring suggested that an unstable state of genetic material is induced by ultraviolet irradiation. This unstable state stablizes during colony growth as either the mutated or the normal genotype. The action spectra of all mutations studied, as well as cell inactivation, had a maximum at 265 mμ, and the shape of the action spectrum approximates that for nucleic acid absorption. He finds, however, that the dose-curves for mutations and cell killing are of the "single-hit" type in his studies on this organism.

Stapelton *et al.* (178) found that alpha-particles and protons were much more efficient than x-rays in inactivating *Aspergillus* spores, and a change in the shape of the survival curves occurred with increasing ion density. A reversal of these relationships occurs in the case of the production of viable mutants. The type of survival curves obtained could be explained either by the "multiple-hit" target hypothesis or by indirect effects. However, the results of mutation curves produced by x-ray suggest that probably two mechanisms were involved in the production of viable mutations. In another series of experiments Stapleton & Hollaender (176) have demonstrated a direct relationship between x-ray sensitivity of *Aspergillus* spores and their relative water content, and they have also reduced inactivation and mutation by decreasing the oxygen tension during x-radiation. These results also indicate two mechanisms of production of mutations by x-rays, one of which is reduced by removal of water or oxygen prior to irradiation. Indirect effects of irradiation play a major role in both inactivation and mutation in *Aspergillus*, and the active agents are probably decomposition products of water which are produced intracellularly by ionizing radiations. Zirkle *et al.* (202) have made similar studies on air dry *Aspergillus* spores. When plotting survival fraction against dose, the alpha ray curves are exponential while x-ray curves are sigmoid, suggesting an explanation based on the "one hit" and "multiple hit" hypotheses, although two inactivating and mutating actions, one caused by the "one hit" effect and the other by indirect effects would also explain the data. Moos (139) finds the indirect effects of ionizing irradiations quite as effective on bacteria. Like fungus spores, *E. coli* and *Pseudomonas aeruginosa* in the dry state are much less affected by x-radiation than when they are in water suspension. However, the high protective effect of dehydration does not appear to result entirely from elimination of the indirect effects of x-radiation.

The indirect inactivation of T-group bacteriophages by x-ray involves at least two different agents, one of which is short-lived and effective only during actual exposure of phage to the radiation, while the other is stable and will act for a considerable period of time [Watson (191)]. Inactive particles produced by indirect effects of x-rays differ in their properties from

phage inactivated by the direct effects of x-rays or ultraviolet. Alper (4) found that the rate of phage inactivation, after exposure to irradiation, was higher than that of phage treated with that amount of hydrogen peroxide which was produced by the ionizing radiation. Pre-irradiation of the phage suspending medium greatly increases the action of direct irradiation, suggesting the formation of free radicals from the peroxide. Buzzell & Lauffer (27) have shown that phage inactivation by x-ray at least in part is due to free radicals formed in the suspending medium. In their experiments inactivation was an exponential function of dose over a wide range when the virus was suspended in 4 per cent broth, but, when irradiation was carried out in 0.8 per cent broth or saline, nonexponential inactivation curves resulted. Moreover, survivors from the 0.8 per cent broth or saline suspensions were inactivated by heat much more quickly than were unirradiated controls. These results also seem to indicate the formation of free radicals in the broth by the x-radiation. In more concentrated broth, the greater part of these are inactivated or neutralized by organic substances in the broth which are in competition with the phage particles for the free radicals. In the dilute broth the situation is reversed, and the inactivation resulting from indirect effects becomes visible. The chemical nature of the indirect inactivation is borne out by the results of the heating-inactivation tests.

Markert (127) has made a comparative study of the effects of x-rays, fast neutrons, and ultraviolet on the conidia of *Glomerella*. He found no qualitative differences in mutagenic effects (so far as nutritional and morphological mutants were concerned) or changes in relative proportions of mutants associated with any dose of any of the 3 types of radiation used. Ultraviolet-induced nutritional mutants appeared to revert to wild type more frequently than did those produced by ionizing radiations, and Markert suggests that this may indicate that physico-chemical changes induced by ultraviolet are less stable than those induced by ionizing radiations.

Atwood (6) has compared the effects of ultraviolet and x-rays on *Neurospora conidia* by means of a heterokaryon technique. He points out that this technique is extremely valuable since several types of radiation damage (the survival, proportion of homokaryotic cells, and the proportion of nuclei carrying recessive lethals) can be measured simultaneously. X-ray treatment produced entirely different results from those produced by ultraviolet with respect to the above factors. The largest comparative difference was found in the proportion of homokaryotic cells among the survivors. This proportion increased rapidly with the dose in the case of x-radiation but remained unchanged with ultraviolet. Other experiments indicate that ultraviolet-induced damage is nuclear, and Atwood states that, if this is the case, then his experimental results show that the ultraviolet effect can be reversed by an interaction between the affected and unaffected nuclei, while the x-ray effect cannot be so reversed. Such an explanation also possibly could account for ultraviolet-induced mutants of *Glomerella* reverting to wild type at a much greater rate than did those induced by ionizing radiations.

Moos (138) found that a pyocyanine-producing mutant of *Pseudomonas aeruginosa* required a much higher dose of x-rays than was required to produce the same lethal effects on the normal parent strain, and suggests that perhaps this mutant produces less of some enzyme involved in hydrogen transfer than does the parent strain. This view is supported by the experiments of Mefferd & Matney (133) which demonstrate that carbon monoxide protects *E. coli* against ultraviolet irradiation and that this is caused by carbon monoxide inhibiting enzymes involved in the transfer of hydrogen to oxygen, thus leaving the cells in a reduced, more resistant condition. These results are just the reverse of those obtained with x-rays by investigators of higher forms; King, Schneiderman & Sax (105) using *Tradescantia* and Haas *et al.* (78) using *Drosophila* have found that carbon monoxide treatment during x-ray irradiation increases the amount of chromosome damage. But when as little as 5 per cent carbon dioxide is mixed with the carbon monoxide, a high degree of radiation protection is attained. Possibly the *P. aeruginosa* mutant produces more catalase than does the parent strain. Clark (34) has shown that *E. coli* mutant strain B/r has a much higher catalase activity than its parent strain, and this probably accounts for the increased resistance of strain B/r to radiation and to the toxic effects of substrates which have been treated with ultraviolet or hydrogen peroxide. Other recent work has emphasized the role played by catalase in radiation protection. Setlow (169) found that the catalase molecule was inactivated in part by x-ray and that its sensitivity to radiation increased as the radiation temperature increased. When three-fourths of the catalase molecule was inactivated the whole molecule became inactive; it appeared that at least two active heme-groups were necessary for the enzymatic activity. Tauber (183) finds that catalase can act as peroxidase but that peroxidases can not act as catalase. Chance (29) finds that the steady state concentration of endogenous hydrogen peroxide in intact *Micrococcus lysodeikticus* cells may be decreased by exogenous donors such as alcohol, nitrite, or formate. Without these donors catalase decomposes hydrogen peroxide slowly. He believes that these results may explain how catalase participates in protection from radiation effects. High energy cathode ray doses sufficient to result in lethal mutations of *M. pyogenes* var. *aureus* did not completely inactivate the catalase activity of this organism [Procter *et al.* (154)]; catalase inactivation in irradiated samples was directly proportional to the post-irradiation storage temperature. Kimball & Gaither (104) found that nongenetic death and division delay in *Paramecium aurelia* by x-ray was caused by peroxides produced in the medium. About two-thirds of the effect could be eliminated by catalase. Latarjet & Caldas (108) and Miletic & Morenne (134) have studied catalase-restoration of irradiated bacteria in connection with lysogenicity. These studies seemed to show that ultraviolet-irradiated nonlysogenic strains are not restored by catalase while lysogenic strains are restored if the catalase is added within 2 hr. after irradiation; restored cells divide at the same rate as normal survivors. Phage production in lysogenic strains infected with T2

phage is restored by catalase treatment following ultraviolet irradiation, while phage production in nonlysogenic strains is not. In *E. coli* strain K12 catalase does not prevent growth of phage and lysis induced by ultraviolet irradiation (Lwoff's phenomenon), and catalase restoration is not found after x-irradiation. Catalase performs its restoring action by destroying peroxides' created by ultraviolet irradiation in the bacterial protoplasm and for some unknown reason this is favored by "prophage." After small doses of ultraviolet, catalase restoration is slight because most of the cells are lysed as a result of Lwoff's phenomenon.

The inability of Saz, Eagle & Toal (166) to obtain an increase in antibiotic resistant mutants when subjecting *M. pyogenes* to ultraviolet irradiation or irradiated substrates may have resulted from their use of high catalase strains. We have been able to induce antibiotic-resistant mutants in seven different strains of this organism by direct or indirect irradiation, but some strains had to be grown in media containing a catalase poison before irradiation treatment in order to obtain positive results. Mefferd (131) also has reported on experiments whose results conflict with those of Saz *et al.* (166).

Radioactive isotopes have been used as mutagenic agents. Hungate & Mannell (93) found radioactive S^{35} to be a very potent and easily handled mutagenic agent for use with *Neurospora crassa* microspores. Their experiments were so conducted as to enable them to distinguish between the effects resulting from transmutation of S^{35} to Cl^{35} and the effects caused by radiation from ionizing β-particles. About 5 to 10 per cent of the total mutants produced were caused by β-particle radiation and the remaining 90 to 95 per cent resulted from transmutation. Furthermore, it appears that only a few genes are involved in the transmutation-induced mutations; but more genes than indicated by the data could contain sulfur and still produce results such as were obtained. Gattani (70, 71) has produced nonsporing mutants and also better penicillin-producing mutants of *Penicillium notatum* by growth on medium containing uranium nitrite. Berk (16) obtained mutants of *Aspergillus niger* by means of the alpha particles emitted from radium and polonium.

Since it has become well established that a considerable part of radiation induced damage is a result of indirect effects of chemicals produced both in the cell and in the substrate, it has become feasible to investigate the possibility of protecting against such chemical action. Bachofer & Pottinger (8) investigated the protection of bacteriophage against x-rays by high concentrations of a neutral salt. They find that this protection is apparently caused by salting out and the resulting dehydration of the phage. This apparently protects the phage only from the indirect effects of x-radiations. Oxygen removal by enzymes [Stapleton *et al.* (177)] and by chemicals [Burnett *et al.* (26)] protect against x-ray inactivation of bacteria. Protective compounds can be divided into two classes on the basis of whether or not preincubation of cells with the compound was necessary for protection. Effective protectors, such as dimercaptopropanol and sodium hydrosulfite, which did not require

preincubation, remove oxygen from suspensions without the need of supplementary enzyme action. A number of the substances which protected the bacteria against the irradiation effects [Whitehead (193)] were thought to act by absorbing out the effective radiation. Mefferd & Campbell (132) compared the influence of temperature on the radiation sensitivity of thermophilic and mesophilic bacteria, and found that thermophiles are more sensitive to radiation killing but that this is less affected by temperature changes than it is in the mesophiles. No temperature effect on radiation sensitivity in the thermophiles was observed when oxygen was excluded, but the mesophile was markedly affected in such experiments. It was more difficult to induce mutations in the thermophile than in the mesophile in regard to drug-resistance and biochemical characteristics.

Evidence has been obtained which indicates that at least the greater part of bacterial killing or inactivation by radiation cannot be regarded as resulting from true lethal mutations but probably is caused by inactivation or destruction of an essential enzyme. Aldous & Stewart (2) reported that ultraviolet inactivation of yeast cells roughly parallels the inhibition of either hexokinase, carboxylase, or zymase. They also found that catalase, alcohol dehydrogenase, or lactic dehydrogenase showed no decrease in activity even when 95 per cent of the cells had been killed. X-irradiation of resting *E. coli* cells completely prevented them from adaptively synthesizing formic hydrogenlyase, but had no effect on the preformed enzyme [Billen & Lichstein (19)]. Rice (160) suggested that radiation mortality resulting from ultraviolet irradiation is related to its effect on the pentose moiety of nucleic acid and related compounds which may have some bearing on Bartholomew & Mittwer's (9) observation that such irradiation caused gram-positive organisms to become gram-negative.

Heinmets *et al.* (85) have studied the photosensitized inactivation and reactivation of *E. coli*. They found that dye binding by bacteria is primarily ionic and that the optimum methylene blue concentration for photosensitized reactivation is 10^{-5} M. Photosensitized inactivation takes place in both frozen and liquid cultures and requires the presence of oxygen. Following the photosensitized inactivation the organisms lose their ability to reduce methylene blue in the presence of various substrates. They are reactivated by prolonged storage at 37°C. in the presence of hydrogen donors.

Beckhorn (11) in studying ultraviolet killing and photoreactivation in *E. coli* strain B/r found the most striking effects during the lag phase. The lag phase was appreciably lengthened by as little as 500 ergs per mm.[2] of ultraviolet, but affected only slightly more by higher doses. This increase in the lag-phase was almost completely inhibited by exposing the ultraviolet-irradiated cells to white light, and the reactivating light had no effect on unirradiated control cells. Neither the ultraviolet treatment nor the reactivation treatment was found to effect the generation time during the logarithmic growth phase. Bacteriophage irradiated in the dry state could not be photoreactivated [Hill & Rossi (88)]; the ultraviolet survival curves

were the same in the dry state as in the wet state, but photoreactivation occurred only when moisture was present. Dulbecco & Weigle (54) have investigated photoreactivation of the induction to lyse produced in lysogenic bacteria by ultraviolet and x-rays and concluded that this was entirely different from photoreactivation of ultraviolet-inactivated bacteria.

Chemical mutagens.—Demerec *et al.* (47) and Demerec & Hanson (46) found that washing cells in a hypotonic solution of sodium chloride, potassium chloride, calcium chloride, sucrose, or dextrose decreased the mutagenic action of subsequent treatment with manganese chloride, whereas washing in hypertonic solutions of these compounds increased it. Temperature during washing had no effect. The inorganic salts could be arranged in the following order on the basis of stimulating the action of $MnCl_2$: NaCl, KCl, $CaCl_2$, $BeFl_2$, UO_2Cl_2, $CdCl_2$, $CrCl_3$, $CoCl_2$, $MgCl_2$ and $ZnCl_2$. The presence of sodium phosphate buffer in the $MnCl_2$ treatment solution entirely suppressed the mutagenic action of the latter, and NaCl inhibited it, but glucose either had no affect or under certain conditions stimulated it. The degree of mutagenesis was greater in resting than in growing suspensions, greater in organisms grown in nutrient broth than in mineral salts medium, greater when treated at high temperatures (37°C.) than at low temperatures (1°C.), and proportional to the concentration of $MnCl_2$ (up to 0.005 per cent) and to the length of treatment up to 30 min. By use of the radioactive isotope Roberts & Aldous (161) demonstrated that the mutagenic action of $MnCl_2$ depends on the cell's uptake of this compound and suggest that the genetically active component of $MnCl_2$ is bound to specific sites within the bacterial cell. The data indicate that $MnCl_2$ acts on the gene indirectly by first inducing changes outside the gene system. These changes affect metabolic activity and gene stability, thereby increasing mutation rate. The suppression of colonial variation in *Brucella abortus* by metal-complexing compounds as reported by Cole (36) is reversed by certain metallic salts including those of manganese and magnesium. While this is not a simple phenomenon the known mutagenic action of manganese salts may offer at least a partial explanation.

Formaldehyde, which previously had been found mutagenic for *Drosophila* and *Neurospora*, is also a powerful mutagen for bacteria [Englesberg (59)]. This critical study leaves no doubt that mutations are induced by formaldehyde in *Pseudomonas fluorescens* which permit the organisms to use itaconic acid as a sole source of carbon. The formaldehyde also increased the end-point mutants of *E. coli* to T1 phage under conditions where growth and selection were excluded. Certain media reactivate formaldehyde-treated cells, and a phenomic lag in the expression of the induced mutants was apparent.

Stevens & Mylorie (179) evaluated the mutagenic action of numerous compounds related to the nitrogen mustards for effectiveness in inducing reversions of biochemical mutants of *Neurospora*. Aliphatic monofunctional mustards were more active against the adenineless mutant and less active

against the inositol-less mutant than corresponding bifunctional compounds. Aromatic substituents in the mustard molecule reduced activity and the 2-chloroethyl vesicants were without mutagenic action. The sulfonium compound thiodiethylene *bis* (*bis*-2-hydroxyethyl) sulfonium chloride found in aged solutions of mustard gas was a very effective mutagen. The mutagenic action of menadione (2-methyl-1,4-naphthaquinone) was confirmed in tests for biochemical mutants of the B.C.G. strain of the tubercle bacillus [Panisset *et al*. (148)].

The chemostat [Novick & Szilard (144, 145)] seems admirably adapted to measuring mutagenic and antimutagenic action. Mutation of a stationary population of *E. coli* in the chemostat to T5 phage resistance occurred at a rate of 1.4×10^{-8} per bacterium per hr.; note that this was independent of the generation time of the organism. Theophylline (a dimethylxanthine) increased this rate tenfold; the addition of guanosine completely suppressed the induced mutations. Guanosine did not select against the mutants since they continued to increase in the population after the addition of guanosine but at a rate only as fast as that occurring spontaneously. The antimutagenic action of guanosine was observed about 12 hr. after its addition because of late expression of mutants. The normally occurring ribosides, adenosine and inosine, had only about one-half the antimutagenic activity of guanosine; ribose, the free purines, and xanthosine were without activity. Guanosine suppressed the spontaneous mutation rate as well as the mutagenic action of theophylline, caffeine, theobromine, paraxanthine, and 8-azaguanine, but it had little effect on the action of tetramethyl uric acid or benzimidazole.

Oxygen and oxidized compounds have a marked effect on the spontaneous and induced mutation rates; consequently, anything affecting the concentration of these substances in the cell would be expected to alter mutation rate. While no work has been reported on the effect of free oxygen on microorganisms, Conger & Fairchild (37) found that chromosomes in the dry pollen grains and microspores of *Tradescantia* were broken at a much greater rate upon the exposure of these cells to increased oxygen tension. Exposure to an atmosphere of pure oxygen produced as many chromosomal abberations as approximately 1200 r of x-rays; it was suggested that the spontaneous breaks might be in part a result of the oxygen present in air. Alexander & Fox (3) suggested that ferrous sulfate, etc., protect against radiations *in vivo* by completely removing perhydroxyl radicals that are formed in the presence of oxygen, and it is the reaction of these radicals with the cell constituents that accounts for those biological effects of irradiation that can be alleviated by ferrous sulfate, thiouracil, allyl thiourea, cyanide, and azide. Whitehead (193) interpreted his results to indicate that a layer of pyruvate protects against the mutagenic action of ultraviolet by simple absorption, but it appears evident from the work of Heinmets *et al*. (85) that the effect of pyruvate on ultraviolet damaged cells is considerably more than one of screening.

Jensen *et al*. (97) concluded that reversion of adenineless *Neurospora*

to wild type would serve as a useful measure of mutagenic action. In addition to the mustards, diazomethane, peroxides, and formaldehyde were effective mutagens while ethyl urethan, the phenols, and ethylene oxide gave negative results. A combination of formaldehyde and hydrogen peroxide induced more mutations than any other treatment. It was suggested that the free radicals whose formation appears to accompany mutagenic action react with compounds in or near the gene, liberating sufficient energy to switch the gene from one allelic form to another. One is unable to speculate on the coupling mechanism necessary for energy to produce such a switch until something is known about the nature of the difference between allelic forms.

SEXUAL RECOMBINATION IN BACTERIA

The new developments in bacterial recombination are presented in detail by Lederberg et al. (113). Genetic recombination in E. coli K-12 appears to be stimulated by supernatants of washed cultures [Maccacaro & Booth (124)], but this may be apparent rather than real because of growth of the organisms in the supernatant. Broth cultures of a streptomycin-sensitive biochemically deficient strain of K-12, treated with sufficient streptomycin to sterilize and mixed with a streptomycin resistant strain with other biochemical deficiencies, still yielded prototrophs which were streptomycin resistant [Hayes (83)]. The reverse cross did not work, suggesting that one strain acts as a donor of genetic material and the other as a recipient; it is suggested that genetic elements extruded by the cell adhere to the cell wall of the killed organism which serves as a passive carrier of the genetic elements. Rothfels (162) and Fredericq & Betz-Bareau (65) presented further evidence of gene linearity in the K-12 strain. Lederberg (109), by measuring the number of papillae produced per colony of nonlactose fermenting mutants of E. coli when grown on a lactose medium, differentiated a number of lactose-negative mutants on the basis of the back mutation rate. By backcrosses to wild type these papillae could be demonstrated to be composed of true reversions. However, mimic reversions were also encountered but these were less vigorous fermenters than wild type. Stable lactose-negative strains may carry a second mutation which lowers the selective value of any lactose positive mutants that might arise. The lactose negative locus could be divided into two components. The relative lack of fertility in crosses between different strains of E. coli may be explained by the discovery of self-incompatible stocks in K-12 containing a mutation designated as F^-. A cross of two F^- strains is completely infertile; $F^- \times F^+$ and $F^+ \times F^+$ are fertile, but the latter combination is less productive, and there appears to be a gradient of relative potencies among the F^+ stocks [Lederberg et al. (111)].

Zinder & Lederberg (201) introduced the term "transduction" to describe the mechanism of genetic exchange in Salmonella. When these organisms were grown in the presence of deleterious agents they released into the medium a filterable agent (FA) that was capable of transferring hereditary traits from one strain to another. Individual filtrates may transfer many

traits but no more than one to a single bacterium. Berry *et al.* (17, 18) transduced multiple nutritional requirements from auxotrophic strains of *Salmonella typhimurium* to other strains by means of sterile filtrates. Not all strains were receptive to the activity of a given filtrate. The wild type allele could be transduced also in this manner. The traits acted independently and appeared to be assorted at random among the recipient cells. Booy & Wolff (22) induced Vi-antigen in typhoid bacilli by growth in the presence of killed Vi-antigen producing bacteria.

The bacterial transformation reactions were reviewed by Austrian (7), and Ephrussi-Taylor (61) interpreted the capsular transformation in the pneumococcus as a variety of genetic exchange in which it is possible to obtain a series of intermediate capsular characters in addition to the complete change in type. Already there have been described at least seven transforming agents which can be recognized in the nucleic acid of lysed pneumococci. Those concerned with penicillin resistance [Hotchkiss (90)] yield a stepwise increase in resistance in the culture into which they have been introduced which may or may not have genetic significance [Hughes (92)]. Whether or not transduction should be regarded as an example of the transformation reaction with the modifications expected from the use of different species can not be decided at the present time. The FA[2] is a much larger particle than the largest estimate offered for the transforming principle [Fluke *et al.* (62)]. By use of graded membranes the former was determined to be of the order of 0.1 μ, while the latter, as determined by bombarding with ionizing particles, appears about ten times smaller. The transforming principle is released on the dissolution of the donor strains while FA is produced during abnormal growth of the donor strains. Most important is Zinder & Lederberg's observation that unlike FA the transforming substance is inactivated by desoxyribonuclease.

CYTOLOGY

In *Tetrahymena* Elliot & Nanney (57) obtained cytological evidence of conjugation. It is hoped that improved cultural methods will permit survival of the conjugants and thus open the way for genetic studies with this organism. Danielli (38) and Ord & Danielli (147) enucleated *Amoeba proteus* to study the relative effects of mutagenic agents on the nucleus and cytoplasm.

The nuclear changes in dividing bacteria are being studied in a number of laboratories. Witkin's evidence (196) that the Feulgen-positive staining bodies carry the genes has already been mentioned. Bisset (20) described the aspects of cell structure of particular interest to microbial genetics, elucidated by his staining procedures, and emphasized differences in form and behavior of the bacterial nucleus from those of other cells. By serial photomicrographs of growing microcultures taken with a phase microscope in dark contrast, Knaysi (106) was able to demonstrate the structure, division, and fusion of nuclei in a *Mycobacterium*. Changes in nuclear structure

in multiplying cells of a variant of *Bacillus anthracis* were observed by a similar technique [Clifton & Ehrhard (35)]. Kellenberger (102) used an improved procedure for studying the nuclear bodies in *E. coli* with the electron microscope. The abnormal behavior of the nuclear material in *E. coli* exposed to ultraviolet irradiation was followed by Delaporte (42). A new nuclear stain was employed by Chance (30) to show sequential development and division in *Gaffkya tetragena*. DeLamater (43) and DeLamater & Hunter (44, 45, 94) have presented evidence for the occurrence of true mitosis in representatives of several major bacterial groups. They have observed conjugation tubes in *Bacillus megatherium* with suggestion of the transfer of nuclear material from one cell to another. The spores were found to have centrally located nuclear material which exhibits a characteristic behavior during sporulation and germination. While there is no unanimity as to the details in nuclear behavior in microorganisms, the general agreement that there are details to observe and a variety of methods for observation promises well for future progress in this field.

Mutant Studies

Bacterial mutants.—As might be expected, the mutations to antibiotic resistance have received considerable attention. The importance to the physician and public health worker of fundamental studies on bacterial genetics and drug resistance was emphasized by Davis (39) together with a discussion of recent applications of genetic principles to this problem. Among the pathogenic staphylococci isolated by Charbert & Terrial (32) many are now found that are resistant to all five antibiotics employed in the clinic. This picture has changed following the introduction of each of the antibiotics. The resistant types predominate in urinary and fecal isolations but they are also isolated frequently from pulmonary lesions.

Bacterial resistance to erythromycin [Haight & Finland (81)], neomycin [Iverson & Waksman (95),] and subtilin [Jann *et al.* (96)] has been studied, and Casida & McCoy (28) developed resistance in the mold, *Candida albicans*, to an unidentified antibiotic. On the basis of the number of genetic steps apparently required for the development of complete resistance, most of the antibiotics resemble penicillin. The general lack of any cross resistance usually observed may result from lack of sensitivity of the methods, and this problem has been studied in detail with *E. coli* by Szybalski & Bryson (182) using the gradient plate technique. Bacteria resistant to one antibiotic may show resistance to other antibiotics equal to the wild type, they may be more resistant (cross resistance), or they may be more sensitive (the term "collateral sensitivity" is suggested for this). An example of the latter phenomenon is when strain B/r of *E. coli* in becoming 300 times more resistant to chloromycetin simultaneously developed a hundredfold increase in sensitivity to polymyxin B. Haight & Finland (81) observed collateral sensitivity to neomycin in erythromycin-resistant strains. Exploration of cross resistance patterns by Szybalski and Bryson showed that the antibiotics fall into four

internally related major groups: (a) streptothricin, viomycin, vinactin (actinomyces polypeptides), catenulin, and neomycin (organisms resistant to these are also resistant to the streptomycins but the reverse is not true); (b) aureomycin, chloromycetin, and terramycin (penicillin and netropsin are less closely related); (c) polymyxin B and circulin (cyclic bacterial polypeptides). Strains resistant to these show one way resistance to streptomycin, streptothricin, neomycin, and catenulin; (d) bacitracin. Whether these relationships are general or whether they hold only for *E. coli* strains is not known.

Bacteria can develop only a limited resistance to nitrofurans *in vitro* [Paul *et al.* (149)]. Eighteen nitrofurans can be divided into two groups on the basis of cross resistance. The second class differs from the first by having a carbon atom between the carbonyl group and the terminal group on the nitrofuran side chain. Strains of *E. coli* can be developed that are resistant to high concentrations of amino acids [Gordon *et al.* (76)]. Strains that are resistant to α-amino-*n*-butyric acid are also resistant to glycine and alanine but alanine resistant strains are only slightly resistant to α-amino-*n*-butyric acid. Rasch & Parker (156) failed to confirm the reported enhancement of penicillin sensitivity upon *in vivo* exposure to that drug. Rather they find that the disk assay method yields varying results depending on the size of inoculum on the seeded plates; when organisms are injected into an animal treated with penicillin the numbers recovered and smeared on the assay plate are much fewer than are obtained from an animal similarly injected but untreated. This accounts for the increased zone size. Temperature effects on drug resistance may be relatively complex. The inability of *Bacillus cereus* to produce the adaptive enzyme, penicillinase, at 42°C. renders it sensitive to 100 times less penicillin than when grown at 37°C. [Knox & Collard (107)] since at both temperatures the organisms will grow only when the penicillin level in the region of the cell drops to 1 or 2 units per ml. The fate of any cell inoculated into penicillin-containing medium depends on initial penicillin concentration, available penicillinase and the possibility of adaptive penicillinase formation. Fever therapy in conjunction with antibiotic treatment should be effective (a) by requiring double mutants for continued infection and (b) by suppressing adaptive mechanisms in the parasite.

The problem of the mutational origin of resistant strains already has been considered. Hughes (92) pointed out that the variation in penicillin resistance was in both directions and that organisms of greater sensitivity as well as greater resistance are present in each transfer in a drug-containing medium. He found the range of resistance to be a continuous function rather than a stepwise increase [see also Eagle *et al.* (55)]. The size of the step in increased resistance was limited by the practicable size of inoculum and not by any inherent characteristic of the cell. His conclusion from experiments with staphylococci grown from single cell isolates was that penicillin resistance in this organism is a flexible character behaving in a way not anticipated either by simple fission into two identical units or from the laws of inheritance pos-

tulated for plants and animals. On the other hand, Rowley (163), who also studied the penicillin-sensitive portion of a bacterial population, concluded that this character was under genetic control. He grew irradiated organisms on plates containing a partially inhibitory concentration of penicillin and, after marking the colonies that appeared, sprayed the plates with penicillinase. A small proportion of the new crop that grew after the destruction of the penicillin was more sensitive than the parent strain (the others were "persistors"). Only one of the more sensitive strains developed an additional nutrient requirement, thus refuting the general application of that theory of penicillin resistance which ascribes this phenomenon to greater nutritional independence. Cinematographic records of *E. coli* in the presence of antibiotics show two clearly contrasting types of organisms; one type lyses explosively and the other develops into "monsters" [Pulvertaft (155)]. Even after neutralization of the drug the first forms that grow always develop into the large aberrant forms.

Some progress has been made on the nature of the resistance phenomenon. Aromatic amine produced by some sulfonamide-resistant staphylococci has been identified conclusively as *p*-aminobenzoic acid [Leskowitz *et al.* (115)]. Streptomycin resistant strains of staphylococci and *E. coli* differ from the wild type in showing a much prolonged lag phase [Beljanski (13)]. Ribonucleic acid is accumulated in the resistant strains but the concentration of proteins, orthophosphate, ribose mononucleotides, desoxyribose mononucleotides and desoxyribose nucleic acid are not modified. This is in agreement with the observation of Thompson *et al.* (185) that streptomycin resistant *E. coli* were markedly deficient in ability ot oxidize ribose. Chaplin (31) has isolated strains of *Serratia marcescens* which are resistant to 1000 times as much of a quaternary ammonium disinfectant as are the wild types. Fat stains show the resistant organisms to possess a surface layer of lipid material which can be extracted by fat solvents or digested by lipase. Following lipase treatment resistance is lost. Reversion to wild type occurs rapidly in the absence of the inhibitor, as would be expected since the production of lipid to the extent of one-third of the dry weight of the organism would place a tremendous handicap on the resistant mutant in an inhibitor-free medium. It is presumed that the elaboration of the lipid which is retained on the cell surface gives resistance (*a*) by excluding the antibacterial agent, (*b*) by reacting with it, or (*c*) by rendering the surface capable of withstanding the disruptive surface force of the disinfectant. An unusual opportunity to get at the mechanism of resistance was available to Davis & Maas (40) who had a single compound *p*-nitrobenzoic acid (PNB) which was inhibitory to *E. coli* because it interfered competitively with two distinct enzyme systems, one involving *p*-aminobenzoic acid, and one involving *p*-hydroxybenzoic acid. Other analogues of these two vitamins are available which interfere with each of these specifically. From an analysis of inhibition experiments it is possible to exclude every explanation for resistance except that of an altered enzyme protein in the resistant strain. Another enzyme

modification in a mutant strain is that reported in the ureases and dehydrogenases of strains of *Micrococcus pyogenes* that are resistant to sulfhydryl inhibitors such as 5-nitro-2-furaldehyde semicarbazone (Furacin), mercurials and trivalent arsenicals [Yall & Green (199)]. It is suggested that these enzymes from resistant strains have fewer or no reactive SH groups as contrasted with similar enzymes from sensitive organisms. The absence of SH groups may not alter enzyme activity, yet it could make the enzyme less liable to reaction and thus to inactivation with sulfhydryl reagents. This is a departure from traditional enzyme chemistry where the inactivation of an enzyme by SH reagents was accepted as an indication of the functioning of that group in the enzymatic process. The temperature sensitive pantothenicless mutant of *E. coli* was shown to be another example of a mutant in which an altered enzyme is produced [Maas & Davis (123)]. The enzyme that catalyses the condensing of pantoate and β-alanine in the mutant is excessively heat labile and this accounts for the difference between the mutant and the wild type. The presence of a stabilizer in the wild type was excluded by measuring heat inactivation of mixtures of the enzymes.

When Brucella was transferred in serial cultures in broths containing increasing penicillin concentrations, two different effects were observed [Braun *et al.* (23)]. Rapid population changes, involving the establishment of nonsmooth mutants in smooth cultures, were associated with increasing accumulation of alanine in the medium, and the smooth penicillin resistant cells appeared which presented a different aspect depending on whether they were subcultured in the presence or absence of penicillin. Nonsmooth colonial and antigenic characteristics were observed when these cells were grown in the presence of penicillin, but when cultured in the absence of the drug they retained their penicillin resistance but presented smooth colony types. Thus environmentally-produced temporary modification results in an appearance which also comes from spontaneous genetic changes and adds to the problems confronting the microbial geneticist.

The transformation of S to R forms in *Salmonella* occurs in hypertonic beef broth to which anti-O serum has been added; simple ageing of the cultures in ordinary broth incubated at 37°C. gives the same result [Beguin & Grabar (12)]. The rough forms agglutinate in very acid solutions (pH 2 to 3 while the smooth forms agglutinate at pH 4. Friewer & Leifson (68) isolated a nonmotile yet flagellated variant of *Salmonella typhimurium* which was morphologically and antigenically identical with the motile strain.

In initially smooth cultures of *Brucella abortus*, alanine does not accumulate and population changes do not occur [Goodlow *et al.* (75)] when L-asparagine is the sole source of nitrogen in a synthetic medium in contrast to the situation when D- or DL-asparagine are used. With smooth cultures of *Brucella suis* population changes do occur when L-asparagine is used but this is due to valine accumulating in the medium not to the alanine accumulation observed with D- or DL-asparagine. Furthermore, the accumulation of valine selects for a nonsmooth (mucoid) type rather than for the rough mutant.

Thus the parent type through its metabolism has a controlling effect on future populations in a closed system even though this effect is entirely indirect, and we have in microorganisms a primitive or rudimentary example of a developmental behavior which may be akin to morphogenesis in higher forms.

The exceptional behavior of the smooth to rough variation in the anthrax bacillus has been cleared up by Chu (33) who found that all normal, virulent *B. anthracis* cells possess the genetic potentiality of producing capsules, but the capsules were produced only *in vivo* or under special cultural conditions. When grown on serum agar in the presence of CO_2 the S-R dissociation falls in line with that of other bacteria. On plain agar in air, rough colonies appear when smooth virulent colonies from the CO_2 incubated serum agar are planted, and the converse is also true. The underlying cause of the smooth characteristics of the avirulent anthrax organisms when grown on plain agar in air is not known, but chain length seems to be involved. With staphylococci, Smith *et al.* (173) found high coagulase to be associated with rough variants. Of four variants of *Bacterium tularense*, which could be disguished from colony characteristics under oblique lighting, only one was capable of immunizing a mouse against subcutaneous challenge [Eigelsback *et al.* (56)]. Several of the strains would protect the more resistant rat against subcutaneous challenge, but only one would protect it against intraperitoneal challenge. Here we have an example of an antigenic variation which can be detected by the resistance of the animal and the site of the challenge.

The small colony variants of coliform organisms which often are isolated from urinary infections grew normally when cysteine was added to the medium [Gillespie (73)]. They differed from the usual coliforms in their ability to use inorganic sulfur in the form of sulfate or organic sulfur other than cysteine. Apparently the appearance of L forms and large round bodies in old culture may be associated in some cases with the amino acids in the medium but the amount of glycine or methionine needed to induce the changes was probably larger than that which would accumulate in the cultures; nevertheless, the solution to this problem appears to lie in the biochemical approach as applied by Dienes & Zamecnik (48). Schaefer (167) has clarified the problem of fast growing (eugonic) and slow growing (dysgonic) strains of the bovine tubercle bacillus. The rate of growth of the dysgonic strain is governed by the amount of fatty acid supplied and the rate at which it can be converted into essential metabolite. Variation from dysgonic to eugonic consists of the acquisition of the ability more effectively to metabolize glycerol and glucose. Rare mutants arise in the dysgonic strain and are selected in a medium low in fatty acid but high in glucose and glycerol. Oleic acid-albumin agar without glucose permits rapid isolation of the bovine strain from natural sources. Smooth avian strains are similar to dysgonic bovine strains in that they require fatty acid for rapid growth. Rough avian strains are most frequently observed on media favoring eugonic bovine strains.

A pyrimidine-requiring, and five amino acid-requiring mutants of *Klebsiella pneumoniae* were as virulent as the parental strain when inoculated into mice but purine-requiring mutants were avirulent [Garber *et al.* (69)]. However, virulence was restored by simultaneous injection of purine or by reversion to purine independence. Wahl (188) describes three types of bacteria in their relationship to a particular phage, resistant, semiresistant and sensitive; the latter two contain numerous mutants. The semiresistant organisms are characterized by the reactions to two different phages which permit their differentiation from resistant, sensitive, or lysogenic strains [Wahl & Blum-Emerique (189, 190)]. The semiresistant organisms are able to pass from a refractory to a receptive state in the presence of the phage; this may be the result of mutation. With sensitive organisms, phages are produced only when the calcium concentration is above a certain value and the phosphate concentration is below a certain value; the implication is that mutation to semiresistance may involve a change in the internal calcium and phosphate balance of the organisms, so that in an ordinary medium these ions are not in the proper ratio inside the mutant. Amino acid deficient mutants of *E. coli* are restricted to a greater extent in protein synthesis than in nucleic acid synthesis when grown on minimal media [Sands & Roberts (165)]; amino acid deficient *Aerobacter aerogenes* will not adapt to inositol oxidation in the absence of the deficient amino acid but purine and pyrimidine deficient strains require only ammonium salt for the adaptation [Ushiba & Magasanik (186)].

Yeasts.—Winge (195), in a review of the genetic situation concerning fermentation in yeast, found that all genes so far investigated are inherited in a strictly Mendelian way. All fermenter genes (one for galactose, four for maltose, and three for raffinose and sucrose) show dominance. Wickerham & Burton (194) offered evidence that the paucity of sporogenous yeast cultures may have resulted from the selection of an individual mating type when the cultures were first isolated; to prevent this one must resort to the use of mass inocula and picking and mixing several colonies if plating can not be avoided. Four different genes in yeast controlling the hydrolysis of α-glucosidic sugars are on the same chromosome [Lindegren & Lindegren (119)]. Maltose, melezitose and α-methylglucoside genes are very close together. The small colony mutants arising spontaneously in several species of *Saccharomyces* have a respiratory deficiency which is the result of the inability of the mutant cells to synthesize some respiratory enzymes, in particular cytochrome oxidase [Ephrussi & Hottinguer (60)]. The proportion of these cells in most strains of bakers yeast is of the order of 1 per cent which corresponds to a mutation rate of 2×10^{-3}/ cell/ generation; some strains have even a higher mutation rate. A genetic and nutritional analysis of seven purineless and two pyrimidineless mutants of *Saccharomyces cerevisiae* revealed five genetic types in the former group and two in the latter [Pomper (152)]. The purine auxotrophs could grow only on adenine or hypoxanthine and were competitively inhibited by guanine; the pyrimidine auxotrophs

used uracil, cytosine, 5-nitro-uracil, uridine and uridylic acid. The guanine inhibition indices support the view that hypoxanthine is a precursor of adenine, and genetic data suggest that five steps intervene between hypoxanthine and some unknown precursor, Kilkenny & Hinshelwood (103) observed changes in the growth characteristics of yeasts on union of haploids and segregation of haploids from diploids. After fully training a diploid to various sugars, its growth was always more rapid than that of either of its parent haploids or its segregants. Before training, its performance might be better or worse than its constituent haploids. The authors suggest that, both on mating and segregation, key structures may be lost, and the general organization of the cell changes; although diploids possess greater potentialities than haploids, the more elaborate organization needed to exploit these may be more difficult to establish by adaptive processes. Spiegelman & DeLorenzo (175) studied the effect of prior adaptation on the capacity of segregants of yeast heterozygotes to form galactozymase. Unadapted heterozygotes of the strain used yield a 1:1 ratio of positives to negatives on galactose test plates. However, when the heterozygote is adapted and segregation occurs in the presence of the substrate, all spores in the asci exhibit the positive phenotype and only after seven to twelve divisions of the positive clones in the absence of galactose do half of them revert to negative phenotypes thus restoring the Mendelian ratio. This supports the thesis that cytoplasmic elements necessary for enzyme formation are produced in the presence of the substrate and the dominant gene and are incorporated into the cytoplasm of the spores during segregation. The cytoplasmic elements do not increase in the absence of galactose and are diluted out. The nature of cytoplasmic genes is discussed in detail by Marquardt (128).

Algae.—Only a few reports on algae fit into the scope of this review. Lewin (116, 117) observed the formation of a primary zygote membrane in *Chlamydomonas moewusii* which is neither cellulose nor pectin but which stains with Schiff's reagent. Of mutations which could be induced in this unicellular green alga the majority were slow growing types that did not respond to external organic supplements. Most of the morphological mutants were poorly flagellated, contained large volutin granules, or were odd-shaped; three biochemical mutants were observed, *p*-aminobenzoicless, thiamineless, and one with an impaired photosynthetic mechanism. Sexual crosses were made, and most characters behave as single Mendelian factors. The chlorophyll deficient strain of *Chlorella vulgaris* [Dube (51)] had a block either between magnesium vinyl phaeoporphyrin and protochlorophyll or between the latter and chlorophyll *a*. This could be a true gene mutation or it could result from a defective chloroplast modified by streptomycin. The extensive work of Moewus on *Chlamydomonas* has been reviewed and evaluated by Sonneborn (174).

Fungi.—Fungi other than *Neurospora* are receiving some attention [Thom (184)]. Pridham & Raper (153) encountered no spectacular success in increasing the riboflavin production in strains of *Ashbya gossypii* by selecting

substrains from spores that had been subjected to mutagenic action. Heagy & Roper (84) estimated the desoxyribonucleic acid content of *Aspergillus* conidia by the diphenylamine method and reported that conidia from diploid strains contain double that found in haploid strains. Wild penicillia commonly occur as heterokaryons which enjoy an advantage in growth rate over the homokaryons that can be isolated from them [Jinks (98)]. The ratio of the two kinds of nuclei found in the wild strains varies with the medium, and this variation affords a means of immediate somatic adjustment to a new food supply or of a progressive adjustment to a changing one. This is an excellent adaptation for a saprophyte living on varied and often changing substrates, and it is suggested that in the penicillia the sexual cycle is lost since variation no longer depends on it. Rees & Jinks (159) observed that in the mycelial tips of the heterokaryons of penicillia the young cells contain up to 15 nuclei although the older cells contain only three. It is in the young tips that readjustment of the nuclear ratios of the heterokaryon takes place as a result of a differential rate of division, which must be under cytoplasmic control.

The average number of nuclei in conidia of *Neurospora crassa* was varied from 2.6 on a minimal medium to 6.2 on a complete medium [Huebschman (91)]. The factors responsible are, at least in part, dicarboxylic amino acids. Further examples of suppressor and modifier genes [Mitchell & Mitchell (136); Lein & Lein (114); Haskins & Mitchell (82)] complicate the genetic analysis of mutant strains of *Neurospora*. A suppressor of acetate requirement probably functions by opening another pathway of acetate production. The response of several genes to the same suppressor cannot be considered as proof of allelism. It seems unlikely that a suppressor gene takes over the function of the mutant gene; instead, the gene in the mutant has been revised so that it does not function in that particular environment; therefore, a new mutation is a suppressor if it modifies the environment so as to render the first mutant gene functional. Two allelic tryptophanless *Neurospora* mutants reacted differently to the introduction of a suppressor gene [Yanofsky (200)]. Neither of the strains formed tryptophan desmolase without the suppressor, and in its presence the enzyme was formed by only one of the strains. No major difference could be detected between this enzyme and that produced by the wild type.

A case of maternal inheritance in *Neurospora* was presented by Mitchell & Mitchell (137). The inheritance of the slow-growth character (designated as "poky") appears to be dependent upon its being carried by the protoperithecial parent. This is the only case thus far observed where the phenotype of the ascospores is different in reciprocal crosses. Bonner *et al.* (21) by use of radioactive tracers showed that some niacinless and tryptophanless strains were fully capable of synthesizing small amounts of the specific compounds. Such incomplete genetic blocks were termed "leaky" and this is relatively widespread since seven out of nine mutant loci showed such leakage. The partial functioning of such blocked reactions, at least during some part of

the growth period, suggests alteration of: (a) the specificity of an enzyme leading to a decrease in rate of the catalyzed reaction; (b) the rate at which the enzyme is formed; or (c) the time at which the enzyme is formed.

Pigmentation in *Neurospora* is affected by both genetic and nongenetic factors [Sheng & Sheng (171)]. The four-color asci found in certain crosses can be explained by a major color gene and an intensifier system. Interference with the formation of carotenoid pigments results in greater formation of phytoflouene which is very likely a precursor of the carotenoids; light affects the production of some pigments by its destructive action on others. Mutants requiring tyrosine or phenylalanine will also grow on α-phenylglycine and one *Neurospora* mutant accumulates a considerable amount of this substance [Haddox (80)]. A sudden growth of this organism after a period of several days suggests the slow elaboration of an adaptive enzyme to permit more efficient use of the substance. Once adapted it perpetuates itself only in the presence of α-phenylglycine. A new mutant strain was reported by Doudney & Wagner (50) that gave wild type growth at 35°C. but was strongly inhibited by L-threonine and to a lesser degree by choline. The threonine inhibition is completely overcome by methionine, homocysteine, homoserine, and sulfanilamide; the choline inhibition is relieved by thiamine. Since inhibition is not produced in the wild type by either of the inhibitors of the mutant it seems that wild type contains a controlling mechanism, absent in the mutant, which prevents a reaction involving threonine or choline from proceeding at an excessive rate thereby producing a deficiency in homocysteine. Homocysteine and threonine may be involved in the biosynthesis of thiamine. The histidineless mutants of Haas *et al.* (79) were inhibited by tyrosine and arginine and the relative concentrations of these compounds were critical for the inhibition.

Protozoa.—In *Amoeba proteus* nuclear damage and cytoplasmic damage by mutagenic reagents can be differentiated. The nucleus is ten times more sensitive to mustard than is the cytoplasm but only $2\frac{1}{2}$ times more sensitive to x-ray [Ord & Danielli (147)]. When the cytoplasm is lethally damaged by mustard, cell division does not occur, but when the nucleus is lethally damaged the cells will still divide unless there is concomitant, severe damage to the cytoplasm. A beginning has been made on the genetics of *Tetrahymena* [Elliot & Nanney (57)]. Sonneborn (174) reviewed the evidence for cytoplasmic determination of nuclear survival, behavior, and differentiation in *Paramecium aurelia*, and Beale (10) has reported on the antigenic variation in another variety of this species.

Bacteriophage.—Bacteriophage genetics (other than mutant studies) was established with the theory of independent replication of genetic determinants to account for the reactivation of ultraviolet-inactivated phage by multiple infection and became firmly entrenched when evidence was presented indicating that genetic recombination occurred in bacteriophage. Since that time linkage groups and allelism have been established for certain characters, and recently Hershey & Chase (86) have described a case of

heterozygosis in bacteriophage T2H. This came from a study of the mixed yields of T2H from bacteria infected with r and r+ phage. In such yields there were always a few mottled plaques which on analysis were found to consist of about equal numbers of r and r+ particles. Since r and r+ were known to be due to allelic genes, the particles containing both these markers were termed heterozygotes. No evidence was found that recombinants have their primary origin in the heterozygotes. This discovery furnishes new information concerning the mechanism of phage genetics and also proves to be a valuable tool since the frequency of double heterozygotes provides a measure of linkage independent of recombination tests. These same investigators also found that when bacteria are infected with three kinds of phage carrying unlinked genetic markers a considerable number of the progeny are recombinants having markers derived from all three infecting types. This high frequency of triparental recombination gives important information concerning the sequence of phage multiplication and recombination.

Doermann & Hill (49) have investigated turbidity of plaque halos in bacteriophage T4 and found several different, previously unreported factors influencing this character. Five of these factors at separate genetic loci were studied by means of two-factor crosses with each other and with previously described factors. The data from such tests indicate that nine different loci influencing plaque halo turbidity fall into three linkage groups. The experiment show that the loci are arranged in linear order in the two-linkage groups which could be studied, and also intimate that recombination in one region is not independent of recombination in an adjoining region. In regard to the latter, it appeared that when recombination occurred in one region, it was slightly more apt to occur also in an adjoining region than in a more remote one.

Scholtens (168) has studied genetic recombination in bacteriophage growing in mixed cultures of *Salmonella paratyphi* B and finds that the phages generated in such cultures are of different types formed by recombination of genetic elements from both strains. The characteristics of the phages depend on the "phage type" of the bacterial strains inoculated into the medium. He also reports on the characterization of types of *S. paratyphi* B by a combination of phage reactions and lysogenic properties. Anderson & Felix (5) in studies on the variation in Vi-phage II of *Salmonella typhi* reported that the presence in a bacterial cell of one type of phage induces a specific phenotypic variation in another, apparently unrelated phage. This, however, seems to be another case of genetic recombination.

Dulbecco (52) designed experiments to test critically Luria's recombination theory of multiplicity reactivation of ultraviolet-inactivated phage. Former experiments to test this theory were based on measurements of the probability that bacteria infected with more than one inactive phage particle (multicomplexes) yield active phage, but these measurements were limited technically to a determination of survivals higher than 10^{-3}. This limitation prevented a critical test of the theory. As a result of new techniques (adsorp-

tion of phage to bacteria in buffer instead of nutrient medium, and superimposing photoreactivation of phage on multiplicity reactivation) the quantitative analysis of the theory has been extended. The results of these experiments are not compatible with the theory of reactivation by recombination of undamaged units from two or more inactivated parent particles. However, Dulbecco points out that the discrepancies which make the theory untenable occur principally at very high doses of ultraviolet, and it may be that the recombination theory describes the basic mechanism but that the mechanics are complicated by other factors arising at high ultraviolet doses. Dulbecco (53) designed experiments to determine if the phage whose nucleic acid is broken down is able to contribute genetic markers to progeny. Superinfecting phage was excluded in 50 per cent of the bacteria when the interval between infections was 1 min. Ultraviolet irradiated T2 will stimulate the host to exclude superinfecting phage at about the same rate as active phage, and when active phage and inactivated phage are added simultaneously to the bacteria the inactive phage will not exclude the other. When both the primary and the superinfecting phage were irradiated with ultraviolet and the ability of the superinfecting phage to cooperate with the primary in multiplicity reactivation was measured, it was found that this ability dropped off at the same rate at which the stimulation to exclusion was manifested, and evidently the superinfecting phage could contribute genetically to the progeny. French et al. (67) have approached this same problem by studying the contribution of phosphorus from isotopically marked irradiated and unirradiated phage to progeny of primary infecting T2. They found that exclusion of superinfecting T2 phosphorus parallels superinfection breakdown; P^{32} transfer was still prevented when superinfection breakdown was suppressed. Therefore they believe that another mechanism, in addition to breakdown, must be postulated for the phosphorus exclusion, and that the two processes are not necessarily related.

Luria (121) has used frequency distribution of spontaneously occurring phage T2 mutants as a tool for determining the mechanism of phage reproduction. His method is based on the following theory: phage mutations occur only in the intracellular state during phage replication; if a mutation occurs then one or more mutants will be liberated, and the actual number liberated will depend on the mode of replication; if gene replication of the character being observed takes place by means of independent successive replications, each of which is controlled by the initial gene, and if the initial gene mutates while turning these out, then all replicas produced after the mutation will be mutants. The mutants would thus be in clones, and the frequency of clones of different sizes would be uniform (since mutation can occur at any stage in the process of replication). However, if replication is exponential and one gene produces n genes, each in turn gives n genes, then if mutation occurs, the frequency distribution of mutants is the same as that observed for bacterial mutants in a series of similar populations. The frequency distribution of phage mutants will then be that of the mutant clones among a large

number of bacterial yields of phage. This analysis of clonal distribution suggested the exponential rate of gene reproduction rather than independent successive replication. This means that the initial gene pattern of the original infecting virus is not needed for every phage replica turned out and that its copies can themselves be sources of replication. It is suggested that recombination occurs late in reproduction of the genetic material of the phage, that recombinants detectable as mature particles around the middle of the intracellular growth period do not reduplicate as such, and that reproduction of the genetic material takes place by reduplication of elements not in the form of mature phage particles.

Watson (191) found that indirect inactivation of phages is caused by at least two different agents. One of these was short-lived and detectable only during irradiation, and the other was fairly stable and detectable by its action after irradiation. Phage inactivated by the short-lived agent had reduced ability to be adsorbed by bacteria, and phages surviving this agent were also less able to be adsorbed. The phages inactivated by the stable agent do not have reduced ability to adsorb, and many of the particles can still kill the host bacterium. These are able also to be reactivated by multiple infection. Photoreactivation has not been found for either case. The inactive particles produced by the indirect effects differ in many proprerties from those inactivated by direct x-ray or ultraviolet irradiation.

A great many attempts have been made to induce mutation in bacteriophage, but generally every method by which mutation is induced in higher forms only succeeds in inactivating the phage particles. Spontaneous mutants of many types may be isolated but the possibility exists that all loci that can be studied in bacteriophage and other viruses may be similar to certain loci in bacteria which are not affected by mutagens. Possibly modification of the host enzymes are necessary before a phage could be manifested as a certain type of mutation. This theory is obviously not currently amenable to experimental tests, but it has received some support recently. Luria & Human (122) have reported on a nonhereditary host induced mutation in phage T2 and T6. Certain B/4 (*E. coli* B resistant to phage T4) mutants of *E. col.* B, when infected by either T2 or T6, liberate mutant phage designated as T*. This T* phage will not multiply in young *E. coli* B cells or those of its mutants but will do so in old and starved *E. coli* B cells. However, in the latter it gives rise only to normal T phage of the corresponding type. T* can be propagated serially as such only in a small fraction of old cells of B/4. The T* particles will also infect *Shigella dysenteriae* strain Sh giving rise to normal T progeny. Furthermore, the T*2 form produced in B/4_0 and B/4_{00} (mutants of B/4) cannot be detected on strain B or B/4 but only on *Shigella dysenteriae* strain Sh. Apparently T* is an imperfect form of the phage which results from an inadequate supply of an essential component in the strain B/4_0 host. This prevents the phage from performing some step needed for production or liberation in young *E. coli* B or its mutants. In the competent cells of strain Sh and of old strain B cultures the defective activity may not

be needed or may be needed at a much lower efficiency level than in growing cells.

Murphy (141) has reported that repeated transfers in 5 per cent peptone or asparagine media of sensitive *Bacillus megatherium* cultures containing C phage have made possible the isolation of lysogenic bacteria which produce different strains of phage. Four distinct plaque types with both clear and turbid analogues can be isolated. This appeared to be simply a case of recombination of the parent phage and some of its mutants; however, no success was obtained in an attempt to isolate a wild type and a phage carrying two mutations when crosses were made of two of the phages, each differing by one mutational step. Murphy believes that the situation occurs as a result of the failure of the C virus to make proper adjustments to the lysogenic condition, while those of its variants which can succeed are selected.

In a recent review Smith (172) discussed the probabilities of new virus diseases being caused by latent viruses. Such latent viruses have been shown to occur in mice, caterpillars, bacteria, and plants, and some of these can be stimulated to activity by various methods such as serial passage in mice, feeding simple chemical compounds, or simply adverse conditions or irradiation. Magil & Jotz (126) have reported an evolution of the influenza virus during the past 18 yr. One hundred strains of influenza A and B viruses isolated at different times during that period were tested by hemagglutination-inhibition tests with six antisera representing six different antigen complexes. Antigenic complexes dominant in the strains which were isolated earlier were missing in those which were isolated more recently and, complexes which did not appear in the earlier strains were dominant in the recent strains. These reports would certainly suggest that genetic recombination and mutation of virus play an important role in viral evolution and epidemiology.

Bacteriophages are very effective selective agents for certain bacterial mutants and as such are probably important in epidemiology. Hewitt (87) and Freeman & Morse (66) have reported on phage action on *Corynebacterium diphtheriae*. Many strains of *C. diphtheriae* carry phages which are capable of lysing other strains, and virulent strains have been isolated as the phage-resistant variants of nonvirulent strains. Certain avirulent strains of *C. diphtheriae* which are not resistant to bacteriophage yield virulent strains when attacked by the phage. This was shown not to be caused by the release of preformed toxin by cell lysis. Mayr-Harting (130) has reported on the importance of bacteriophage as the selective agent in epidemics caused by *Shigella sonnei*, and Fredericq (64) has reported similar studies on infections caused by *Salmonella schottmuelleri*.

The following reports do not deal with bacteriophage genetics but are included here because of their close relationship to the subject. Wyatt & Cohen (198) reported the isolation of a new pyrimidine base from bacteriophage nucleic acids. They find that the even numbered T phages contain 13 moles of 5-hydroxymethylcytosine per 100 moles of nucleotide. Wyatt (197) in a study on the nucleic acids of insect viruses found that the ratios of

adenine to thymine and of guanine to cytosine are constant and close to unity in 11 different viruses while the ratio of adenine+thymine to guanine +cytosine ranges from .71 to 1.81. He found no general parallelism between this ratio and biological relationship of the viruses. Miller, Eitelman & Golder (135) suggested that a mutation has occurred in their strain of the southern bean mosaic virus; when first isolated this virus had an isoelectric point of 5.5, and now it is 5.9. The subject of lysogenesis or lysogenic bacteria, insofar as it is concerned with bacterial recombination and transformation, was regarded as being beyond the scope of this review, and the reader is referred to an eclectic construction of the field of genetics and hereditary symbiosis by Lederberg (110).

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DEVELOPMENTAL STAGES OF VIRUSES[1,2]

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INTRODUCTION

Study of viral development is hampered by the fact that viruses defy definition as a biological class. Even so broad a characterization as Luria's operational definition of a virus as an "exogenous submicroscopic unit capable of multiplication only inside specific living cells" (1) underlines our limited powers of recognition in that we fail to detect multiplication, unless the virus is also capable of producing some detectable abnormality in a host organism. This may, however, not be essential to the continuity and survival of the viral species. Dependence on pathogenicity as an index of viral activity is quite out of step with our knowledge that viruses are subject to mutations and can undergo "adaptive" variation from avirulence to virulence and vice versa. In the light of this, how are we to distinguish, under some conditions, avirulent variants from viruses at intermediate stages of development?

The dilemma is illustrated by the example of lysogenicity. If multiplication in the individual host cell were to be the criterion for "maturity" of the infecting viral particle, then a particle setting up lysogenicity might be called "immature." Yet, its propensity as a "mature" infectious and multiplying agent is fulfilled when it gives rise, whether by intracellular mutation as postulated for some systems [Boyd (2)] or by some metabolic modification of the host-virus equilibrium [Lwoff *et al.* (3)], to a crop of virus capable of lysing sensitive cells. That the symbiotic infection can be recognized at all in such systems is in part a result of the rapid rate of multiplication of bacteria which permits the easy detection of a chance event, i.e., liberation of free phage in a lysogenic culture.

In other host-virus systems, whether animals, insects, plants, or tissues derived from them, events of comparable frequency would be extremely hard to detect. Yet the problem of "virulent" and "symbiotic" viral variants is obviously relevant to a discussion of developmental stages of viruses. Does the "masking" of virus in virus-induced tumors result from arrested viral development, or is it an expression of a hereditary symbiotic equilibrium between a viral variant and its host cells? In several other instances, recovery of "noninfectious" virus from infected tissues has been interpreted as being

[1] The survey of the literature pertaining to this review was concluded in January, 1953.

[2] The following abbreviations are used: C-F for complement-fixing; DNA for desoxyribonucleic acid; EID for egg infective dose; HA for hemagglutinins; NA for nucleic acid; RNA for ribonucleic acid.

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attributable to incomplete development. Here, again, the virus may well be complete in the sense that each unit has reached its ultimate stage of development, but "incomplete" phylogenetically in that it deviates from the arbitrarily defined species norm.

These general considerations are important in that they lay down a basic premise which will guide this review: in speaking of developmental stages of a virus, one must have at least one reference point clearly defined, i.e., the finished product. To be dogmatic about it, one should, further, be able to show or have reason to assume that each individual viral unit goes through that stage at some time during its genesis. These two conditions are fulfilled in very rare instances, and many claims for the positive identification of developmental stages are debatable.

Facts and speculation have come from many and diverse sources and have found recent expression in many reviews and symposia which should be consulted for detailed analyses, specialized viewpoints, or for stimulating flights of the imagination far more luxuriant than the purpose or allotted space of this review permit. Several recent symposia (4, 5, 6) have gone far in bringing together the viewpoints of workers in different disciplines of learning and in bringing into sharper focus the relative advance of knowledge in the four major categories of virology. i.e., virus infections of higher animals, insects, plants, and bacteria.[3] The important relationship between viruses and cellular genetics and hereditary symbiosis has been reviewed by Lederberg (7).

In attempting to organize the material, the problems may be summarized as follows: (a) The usual infectious cycle involves extraneous viral particles and susceptible host cells. The first clue to the genesis of viral progeny is the fate of the infecting particle itself; viral development is assumed to occur throughout the interval between first contact and final emergence of new virus. (b) Infectious virus arises or seems to arise in host cells not previously exposed to extraneous virus. Here, the concept of viral precursors or "proviruses," integrated into the genetic apparatus of the host cell or firmly associated with it, has received fresh impetus from the important studies of Lwoff *et al.* (3) on induction of lysis in lysogenic *Bacillus megatherium*.

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[3] Recent volumes of this publication have presented excellent reviews on numerous aspects of viruses and viral infection. They all contain much information which is pertinent to the subject of the present review. But there is hardly one author who can claim equally critical insight into the complex and very different problems posed by diverse host-virus systems. The present reviewer is no exception. He lays claim to first-hand acquaintance with only a few animal-pathogenic viruses. In his treatment of other classes of viruses, especially those affecting plants and insects, he pleads guilty of glaring omissions and bias. Limits of space have necessitated arbitrary selection. The author is grateful to have had the benefit of reading in manuscript form comprehensive reviews by Henle (8) and Bergold (9). Dr. Henle also kindly supplied galley proofs of certain papers presented by speakers at the Conference on Viral Multiplication in Oxford to which reference will be made.

The problem of viral development can be approached in three broad lines of attack: (a) search for biological properties, other than infectivity, which one might think of as possible attributes of developing viral particles; (b) attempts to discern morphological or other physical differences between samples derived from cells at different stages of infection; (c) *chemical* analysis of the host-virus complex and its environment. Clear separation between these three approaches is, of course, impossible if only because the most valuable contributions have been those which have correlated biological with physical or chemical findings. In the following sections, discussion of bacteriophages will precede that of animal viruses, and only scant reference will be made to insect and plant viruses. It should not be inferred from this arrangement that what is known about development of bacterial viruses can necessarily serve as a representative model for the other classes. Recognition is, however, given to the undeniable fact that rapid progress in the phage field has given research on viruses in general much by way of unity of approach.

BIOLOGICAL ASPECTS OF VIRAL DEVELOPMENT

EVIDENCE FOR SYMBIOTIC EXISTENCE OF VIRAL PRECURSORS

Bacterial viruses.—Despite the rapid progress of recent years in the study of lysogenic bacteria, the evidence for presence of viral precursors (prophage) postulated by Lwoff & Gutmann (10), and Lwoff, Siminovitch & Kjeldgaard (3) remains indirect. That is to say, the prophages have not been demonstrated as distinct entities; their reality may be inferred from the observed fact that lysis and liberation of active phage can occur in the progeny of a single bacterium cultured through 19 consecutive generations in the absence of extraneous phage (10), and that it can be induced at will by a variety of procedures. Inducing factors for lysogenic *B. megatherium* and other systems are ultraviolet light (3), x-rays, reducing substances such as thio-malate, thioglycollate, reduced glutathione or ascorbic acid [Lwoff & Siminovitch (11)], hydroxyquinoline [Lwoff (12)], and various mutagenic and carcinogenic agents, among them nitrogen mustard [Jacob (13); Lwoff & Jacob (14)]. From the mode of action of oxine, Lwoff (12) has concluded that induction may be the result of cationic permutation, i.e., replacement of Co^{++} by Cu^{++}, at the level either of the prophage itself or of an enzyme active in the conversion of prophage to phage. Analogous observations on induction have been made on lysogenic strains of *Escherichia coli* K12 [Weigle & Delbrück (15)], *Pseudomonas aeruginosa, Micrococcus pyogenes* var. *aureus* [Jacob, 1950 (16)], *Corynebacterium diphtheriae* (17), and *Salmonella schottmuelleri* (18).

At the same time, it has become clear that inducibility, at least by the methods used by Lwoff *et al.*, is not a general property of lysogenic systems. Bertani (19) was unable to induce phage liberation in *E. coli* strain "Li." Ionesco (20), working with various lysogenic strains of *B. megatherium*, carrying prophages 1 or 2, found that the development and the liberation of

phage 1, but not of phage 2, occurred after irradiation. This was true even for the lysogenic system *B. megatherium* 17[1, 2], i.e., a strain carrying both prophages: only phage 1 was liberated after induction. The complexities of this problem have been further studied by Jacob (21) on "polylysogenic" *P. aeruginosa*. Of three systems, each derivatives of *P. aeruginosa* 13, containing prophages 1, 4, or 8, the latter two were inducible, the former was not. Jacob isolated doubly lysogenic strains 13[8, 4] and 13[8, 1]. In the case of 13[8, 1], ultraviolet irradiation led to lysis and to liberation of phage 8 from more than 95 per cent of the cells while liberation of phage 1 was not increased. In contrast in the system 13[8, 4], one cell lysed spontaneously for every 600 cell divisions. Fifty per cent of those lysing yielded only phage 4; 25 to 30 per cent only phage 8; 20 to 25 per cent both phages. After low-grade irradiation, enough to induce lysis of 20 to 30 per cent of the bacteria, these liberated either phage 4 or phage 8 in a 2:1 ratio. After heavier doses of ultraviolet, however, which induced lysis of 95 per cent of the cells, almost all liberated both viruses simultaneously.

Thus, it seems that each prophage-bacterium system has its own specific equilibrium and that two such systems may exist side by side and display their mutual independence in a single cell. The high degree of variability is evident from unpublished work by Wollman [mentioned by Jacob (21)], who found that when phage liberated from induced *E. coli* K12 set up lysogenesis in indicator strain *E. coli* 122, some of the resulting substrains were inducible while others were not. Another variation has been described by Lwoff & Siminovitch (22): By subjecting *B. megatherium* strain 899[1] to frequent transfers in a synthetic medium, as described by Clarke (23), its lysogenicity was "cured." The resulting strain 899[1d] behaved toward phage 1 like a sensitive indicator strain: cells were either lysed or they became again lysogenic and the latter could be induced. In one of these secondary lysogenic strains, 899[1d] 91[1], induction by ultraviolet irradiation led to lysis without phage liberation.

This would suggest that lysis may not necessarily depend on complete conversion of prophage to mature, infectious virus. It is reminiscent of the close similarity in character and mode of liberation between *E. coli* phages and the "antibiotic" colicine [cf. Frédéricq (24)]. Jacob, Siminovitch & Wollmann (25) showed that the majority of cells in cultures of colicinogenic *E. coli* "ML" can be induced by ultraviolet irradiation, by mutagens, or by carcinogens to lyse and liberate colicine. The biochemical consequences of such an induction closely parallel those seen in induced lysogenic *E. coli* (see p. 106). Is colicine an "incomplete" phage? And is the development of virus in Lwoff's *B. megatherium* 899[1d] 91[1] arrested at an even earlier stage, i.e., before it acts as a nonmultiplying, lytic agent on sensitive indicator cells? That the parallelism between *E. coli* phages and colicines is not an isolated phenomenon is clear from closely analogous findings by Jacob, Siminovitch & Wollmann (25) in *P. aeruginosa*.

It may be said, then, that the equilibrium between prophage and host

cell is perfectly balanced. Experiments on inducing agents have given clues to some factors involved in the occasional spontaneous disturbance of this equilibrium. Jacob (21), in discussing the factors responsible for spontaneous or induced phage liberation, or for noninducibility, concludes that the possibility of mutation of the phage alone does not fit the experimental data. Nor, it is obvious from the data on polylysogenic systems, is the effect on the bacterium per se the determining factor. We are left to conclude that the stability of each prophage-host complex is governed by its own specific metabolic or genetic control device, i.e., that each complex is to be considered as a distinct biological unit in its own right.

This brings the position of the prophages close to the level of gene-linked traits. Lederberg & Lederberg (26) have reported crossing experiments with *E. coli* K12 "which point to a single chromogene as the major determinant of the sensitive, lysogenic, and immune states" (7).

The close link between lysogenesis and hereditary traits of host bacteria is well illustrated by the long-recognized relationship between serological host specificity and lysotype. More recently an interesting correlation has been found in *C. diphtheriae* between toxin production and lysogenicity [Freeman (27)]. Additional evidence will be discussed below in connection with the biological contributions of host cells to actively multiplying viruses.

The genetic unity of the prophage-bacterium complex, evidenced by the studies of the French workers, does not seem to this reviewer to be in essential conflict with the experimental evidence presented by Boyd (2, 28, 29, 30) for the role of independent viral mutation in lysogenic systems of *Salmonella typhimurium*. If reversible differentiation of "symbiotic" and "lytic" viral particles does occur, it could conceivably take place during the vegetative stage of viral multiplication and, in this case, the postulated heterogenicity of the viral yield may have little relation to the factors responsible for the occurrence or nonoccurrence of prophage-to-phage conversion.

Animal viruses.—With methods now available, the development and multiplication of animal viruses can be studied only in association with entire cell populations, not in single infected cells. In this respect, infected tissues are comparable, at best, with bacterial suspensions in their growth medium. The presence of virus gives no hint as to the number of individual cells which are infected, or to the manner in which they acquired infection. The ability of pathogenic viruses to destroy specific cells is often acquired as a result of "adaptation," but that does not mean that the "unadapted" virus may not be able to multiply in the same cells. Conversely, the genetic constitution of the host determines its "vulnerability" [Sabin (31)], but not necessarily its ability to support viral multiplication. Little is known about the way in which virus-cell interaction resolves itself in situations where the cell is not destroyed. Indirect evidence in some instances suggests the establishment of "masked" infection, i.e., an equilibrium state between the virus in a non-multiplying phase and the host cell. The question is whether cells infected in this manner can convey the dormant virus to their daughter cells, i.e..

whether a "pro-virus" can be acquired without exogenous infection. This problem arises in particular in connection with those viruses which produce pathological cell proliferation rather than cell destruction, notably the tumor viruses.

Shope, in reviewing the "masking" of rabbit papilloma and swine influenza viruses (32), defined the problem as follows: "A masked virus ... [is] a living virus which, for reasons that we do not fully understand, has been rendered non-infective and therefore not directly detectable. ... [It is] known by circumstantial evidence or by a series of indirect tests to be present but is not of itself directly demonstrable." Papilloma virus in cell-free filtrates isolated from cottontail rabbits, can induce papillomas in cottontail or domestic rabbits. It can be serially transmitted in this manner in cottontails, but not in domestic rabbits. From the latter, the tumor, although induced by the free virus, can be transmitted only by grafting on other domestic or cottontail rabbits. Only occasionally, very small amounts of virus can be recovered directly [see recent reviews by Ginder (33) and Syverton (34)]. Immunological tests, nevertheless, indicate the presence in domestic rabbit tumors of virus-specific antigen. The fact that tumor-specific cells proliferate in the domestic rabbit in absence of free infectious virus suggests the existence of a true and hereditary symbiosis between virus in a nontransmissible form and its host cell.

Similar findings have been reported for the Rous sarcoma [reviewed by Shrigley (35)]. Filtrability is easily lost although the tumor itself remains transplantable. Shrigley (35) discussed transfer series in which the tumor agent reverted to filtrability after several passages of nonfiltrable lines. Miszurski *et al.* (36) reported that x-irradiation of five nonfiltrable growths with 50,000 r rendered three of them filtrable. Carr (37) reports on quantitative aspects of virus recovery from filtrable fowl tumors. His best yields indicated that only about one cell in twenty contained infectious virus, but that for every infectious particle there were about 570 similar but noninfectious ones, or an average total of about 28 particles per cell. The question is unresolved whether single cells may yield a mixture of infective and noninfective particles or whether infective ones are produced in large numbers in occasional cells only.

The role of carcinogens as possible "activators" of masked viruses or viral precursors has been under much investigation [Rous & Friedewald (38); Rous & Rogers (39)]. The endogenous origin of tumor-producing viruses or, in some cases, the viral etiology of tumors induced by treatment would have to be ascertained before a definitive interpretation is attempted. In other cases, e.g., the role played by fowl pox virus in the induction of tumors in methylcholanthrene-treated chickens [Duran-Reynals (40)], no evidence is available to suggest that the latent virus is present in a noninfectious form.

The nature of other often cited examples of "activation" of latent viruses in man or animals is equally uncertain. Carton & Kilbourne (41) observed herpes simplex in 16 of 17 patients whose trigeminal sensory root was sec-

tioned. In each case, the lesions appeared in the area supplied by sensory fibers on the cut side. They attempted virus isolation from two Gasserian ganglions and failed. Their findings are in accord with those of others. There can be little doubt as to the existence of some sort of equilibrium between herpes virus and its human host, but whether alteration of this equilibrium is conditioned by host factors or by direct modification of the virus is not known.

The same reservations apply to "activation" of other latent viruses in animals, such as pneumonia virus in mice and other rodents [Horsfall and Hahn (42)]. In other cases, where a pathogenic virus is recovered after a series of organ-to-organ transfers in normal animals [cf. Virus III (43)], it is difficult to distinguish between activation of a primarily noninfectious (pro-) virus and adaptive variation on passage of an originally nonpathogenic virus into its virulent form.

The work of Shope (44) on the masking of swine influenza virus in lung worms suggests the existence of the virus in a noninfectious form. Study of the life-long association of many animal- and plant-pathogenic viruses with arthropod vectors [briefly reviewed by Steinhaus (45)] should provide valuable information on possible symbiotic forms of these agents.

Plant and insect viruses.—The bio-assay of plant viruses is not comparable in sensitivity with that of bacterial or animal viruses. Although in some instances good correlation can be shown between virus concentration and number of lesions produced [Bald (46)], the doubt about the number of particles, even in purified virus suspensions, required to induce a lesion (47) makes it precarious to interpret some data on latent infection of plants as suggesting the presence of noninfectious pro-viruses. In relation to the problem of viral development, a decisive distinction has to be made between symbiotic infections in the sense of lysogenesis (i.e., presence of a pro-virus) and latent infections in the sense of a saprophytic carrier state.

This is well illustrated by the story of paracrinkle virus of King Edward potatoes. This agent was believed to reside in King Edward potato plants in an innocuous pro-virus form because it could be detected only on grafting to certain other potato varieties. However, Bawden, Kassanis & Nixon (48) were able to transmit the virus mechanically from healthy King Edward plants to susceptible varieties suggesting that the virus was present in King Edward plants in its infectious form. Whether this is always so, or whether plants may contain pro-virus and mature virus at the same time in varying proportions cannot be decided until the quantitative conditions governing mechanical transmission are more precisely defined. While latency of phyto-pathogenic viruses is common, not only in plants but also in their vectors, "no one has yet succeeded in breaking down the relationship between a plant and a truly latent virus" [Smith (49)].

Latency of insect viruses is believed to be common and may be responsible for chemical "induction" of polyhedral disease in certain insects as well as for the transmission of virus from generation to generation. Bergold (9)

has reviewed the subject exhaustively. Here again, evidence for latent infection does not prove the existence of viruses in a precursor state.

Exogenous Infection: Adsorption and "Disappearance" of Infecting Viral Particles; the Latent Period

This section will deal with the evidence for a "negative" phase in viral development. It is the phase in the infectious cycle, between adsorption of infecting particles and release of new ones, during which there is an apparent or real net loss of viral activity.

Bacterial viruses.—On the subjects of adsorption and viral "eclipse," little can be added to the material reviewed last year by Price (50). The two-step nature of adsorption (51, 52), i.e., a reversible step dependent on cation concentration followed by irreversible attachment leading to "invasion," has been confirmed by Stent & Wollman (53). The beautiful studies of Hershey & Chase (54) on *E. coli* phage T2, have given concrete substance to the negative evidence for the "disappearance" of infecting particles. They have clearly demonstrated the physical separation of the phage DNA[2] from the protein membrane upon attachment. The DNA is believed to be "injected" into the bacterium through the attached phage tail, leaving the membrane outside. Removing the latter from the bacterium by shearing in a Waring blendor has no effect on the independent ability of the DNA to stimulate phage reproduction. The DNA cannot be identified biologically as phage and cannot be released from bacteria. Hershey & Chase conclude that, in the infected bacterium, the phage DNA "forms part of an organized intracellular structure throughout the period of phage growth." The genetically inert protein-containing membrane retains its antigenic identity. Adsorption of phage particles on bacterial debris leads to the same release of DNA. Part of it appears in solution, the remainder can be released from the debris by desoxyribonuclease.

The uptake of phage genetic material by the bacterium sets in motion a chain of events culminating in the release of newly produced viral particles at the end of the "latent" period. The length of the latent period is specific for each system and varies under different external conditions [see Anderson (55), Price (50)]. Premature arrest of viral multiplication [Foster (56)] or premature lysis of infected bacteria [Doermann (57, 58; Anderson & Doermann (59); Kay (60); Joklik (61)] has consistently revealed that infectious particles begin to appear about the middle of the latent period and continue to increase up to the time of spontaneous lysis. Genetic studies, search for "intermediate," i.e., noninfectious forms, and the use of physical tools (microscopy, radiosensitivity data) have yielded clues to some of the events leading up to emergence of mature virus particles. These studies will be dealt with further below.

Animal viruses.—Reliable data on the fate of adsorbed or infecting viral particles depend on the ability to separate cells from their environment after exposure to virus. This ideal can be approached in the case of surface tissues

(cf. respiratory epithelium, allantoic cavity) or in tissue cultures, but not in parenchymatous organs or under conditions permitting systemic spread of virus through the host organism.

In the chorioallantoic membrane of chick embryos, a number of virus infections have been studied in great detail. When influenza virus in amounts ranging from 10^2 to 10^9 ID_{50} is inoculated into the allantoic cavity, an average of 70 per cent of the infectious virus is removed within one hour from the allantoic fluid and presumably adsorbed on the lining endothelium [Henle (62)]. The amount found in allantoic fluid prior to release of newly produced virus from cells may represent an equilibrium state attributable to continuous adsorption and elution of virus not participating in multiplication processes, because it has been found by Isaacs & Edney (63) that heat-inactivated, enzymically inactive virus is adsorbed progressively until all hemagglutinin disappears from the allantoic fluid.

Adsorption of influenza virus appears to be mediated by contact of viral particles with cellular receptors, since their destruction by receptor-destroying enzyme of *Vibrio comma* reduces adsorption by the membrane [Stone (64)]. Enzymic destruction of receptors by the virus does not seem to be a prerequisite for "entrance" into the cells [Isaacs & Edney (65)], although intactness of enzymic function and availability of receptor-like substrate do seem to be essential for multiplication.

Allantoic membranes harvested after adsorption yield only 1 to 5 per cent of the calculated infective virus [Hoyle (66); Henle (62)]. This is true also for membranes infected *in vitro* [Hoyle (67)]. The "loss" in infective virus is reflected equally in loss of viral hemagglutinin [Henle & Henle (68); Hoyle (66, 69); Isaacs & Edney (65); Liu & Henle (70)], complement fixing antigen (66, 68), and other viral activites (65). Inoculation into the allantoic cavity of immune serum (68) during the first hour after infection further reduces by 100 to 1000-fold the amount of infective virus in the membrane without affecting the yield of newly produced virus at the end of a single growth cycle.

The data cited are in accord with the view that those viral particles which give rise to progeny lose their identity upon entrance into cells. The mechanism of this process is unknown. It has been postulated that infecting particles are broken down into subunits [Hoyle (67); Burnet (71)], but in absence of better knowledge this is little more than a play on words. We do not know whether genetic and nongenetic materials of influenza virus separate as they do in the case of *E. coli* phage T_2. It may be significant that heat-inactivated virus particles which are capable of inducing interference likewise "disappear" after adsorption on the chorioallantoic membrane [Isaacs & Edney (65)]. It has also been shown that the suppressed virus in interference experiments "disappears" (65). Hence, the "disappearance" of adsorbed virus, while probably an integral phase of viral multiplication, does not in itself determine whether or not reproduction will occur. With adsorption and "disappearance" of infecting virus, certain levels of all measurable

viral activities are established in membrane and allantoic fluid. These levels remain unchanged for several hours and presumably represent residual injected virus. The length of the latent or "dark" period is characteristic for each strain (68, 72, 73), but varies in eggs of different ages [Freymann *et al.* (74)]. In the case of the Lee strain of influenza type B, it varies with different concentrations of the seed inoculum [Liu & Henle (75)]. Under certain conditions, it has been possible to show that emergence of newly produced infectious virus is preceded by an increase in other viral activities, or that an increase in these activities occurs without commensurate rise in infectivity. These data will be discussed below.

Apparent or actual "disappearance" of virus in early stages of infection of the chorioallantoic membrane has been noted with a number of other viruses: Fowl plague [Schäfer & Munk (76)], meningo-pneumonitis [Girardi, Allen & Sigel (77)], psittacosis [Heinmets & Golub (78)], vaccinia [Briody & Stannard (79)]. Similar findings have been reported in infection of the respiratory epithelium of mice with influenza virus [Fazekas de St. Groth & Donnelley (80)] and pneumonia virus of mice [Ginsberg & Horsfall (81)].

Crawford & Sanders (82) have studied the fate of vaccinia virus in the skin of rabbits. An initial drop in infectious titer was observed, and this coincided with Robinow & Bland's inability to find recognizable elementary bodies in cells during the first stage of infection (83).

As pointed out above, similar observations in other organs have to be interpreted with caution because there is no sure way of determining the proportion of the inoculum which has access to and "enters" susceptible cells. Extra- and intracellular virus cannot be separated, and the extent of systemic spread throughout the host organism is uncertain. Thus, when different amounts ($10^{2.5}$ to 10^8 LD_{50}) of western equine encephalomyelitis virus are inoculated intracerebrally in mice, only 3.5 to 10 per cent of the theoretically recoverable virus can be found in their brains after 1 hr. [Schlesinger (84)]. The same rate of recovery from mouse brain is found after intracerebral inoculation of influenza virus (85) or of bacteriophage (84). Cairns (86) confirmed and extended these findings and reported recovery of the bulk of the "missing" inoculum in extraneural organs. These findings leave the actual baseline of intracerebral multiplication very much in doubt. This applies not only to the reports mentioned but also to similar findings for herpes [Burnet & Lush (87)] and poliomyelitis viruses [Ainslie (88)]. Interesting experiments have been described by Sanders (89) who inoculated GDVII virus intramuscularly and followed the course of infection along the sciatic nerve. Nerve fragments were removed at 10 hr. intervals, and a rate of centripetal migration of 0.2 mm./hr. was calculated. No "disappearance" of infectious virus was noted in the nerve. On the other hand, when the virus was injected intralingually and the hypoglossal nucleus tested for virus at 5 hr. intervals, there was a slight increase at 10 to 20 hr. ("arrival"), followed by "disappearance" between 20 and 60 hr. after inoculation. Masking of virus was inferred from the fact that hypoglossal nuclei removed during

the secondary "eclipse" period, when no active virus could be recovered by subinoculation, nevertheless produced death and specific lesions in mice to which they were transplanted. This interpretation would imply specific intracellular localization of the factors responsible for viral "eclipse." The experimental data thus far shown by Sanders open an interesting approach to this problem; in view of the long intervals between assays used, the unavoidably crude methods of quantitation, and uncertainties regarding the exclusively neural spread of virus, they require extensive elaboration.

Latent periods have been described for every virus-host system which has been studied in sufficient detail. Its duration, in general, depends on the adaptation to the host and may vary in proportion to the over-all growth rate of the virus [Schlesinger (84); Davenport & Francis (90)].

Freymann, Tamm & Green (74) followed multiplication of influenza virus in single eggs and found that often the first rise in infectious virus was followed by one or two further plateaus. These secondary "latent" periods were shorter than the first, a finding confirmed by Cairns (91). According to Fazekas de St. Groth & Cairns (92), the shortening of secondary latent periods does not reduce the relative unit-for-unit yield of virus in consecutive cycles.

As in the case of phages, the events taking place during the latent period are conjectural. It must be assumed that synthesis of virus-specific materials and replication go on during this time. Some evidence in support of this view will be discussed below.

Plant and insect viruses.—The fate of infecting particles of plant viruses has not been determined. This is not surprising in view of the relatively insensitive methods in those cases in which mechanical transmission is feasible. The problem is even more complicated when the infection cycle requires insect vectors or transmission by dodder union [Bennett (93)]. Experiments by Maramorosch (94) have shown unequivocally that aster yellows virus multiplies in its vector, the leaf-hopper *Macrosteles fascifrons, Stal.* It can be transmitted from insect to insect by inoculation. Recent work (95) by the same author indicates that utilization of the insect host may facilitate more sensitive quantitative assays of viral activity. In both insect and plant host, the virus cannot be detected for some time after inoculation. It remains to be seen, however. whether this is related to a "dark" period in viral development, or to inability to detect virus in concentrations below a certain threshold.

Little seems to be known about the fate of infecting viral particles in polyhedral diseases of insects. Bergold (9) reports that one LD_{50} of polyhedral virus by intralymphal injection into larvae of *Bombyx mori* contains about 1000 to 2000 mature single viral rods.

GENETIC ASPECTS OF VIRAL DEVELOPMENT

Bacterial viruses.—The studies of Hershey & Chase on phage T2 [(54) see above] have shown unequivocally that the infecting particle does not

enter the bacterial host cell intact. It remains to be seen whether the phage DNA which does enter reappears as such in the progeny. On the basis of genetic evidence, derived from detailed analysis of spontaneous viral mutation (96) and from the distribution of recombinants at different stages in the growth cycle [Doermann (57)], Luria (97) concludes that it does not. The finding by Dulbecco (98) that the kinetics of multiplicity reactivation do not fit the theory of genetic recombination, leads Luria to revise his earlier hypothesis of independent replication of genetic subunits (99). No clearly formulated mechanism has been substituted for this hypothesis other than that the genetic material is replicated independently and exponentially during the eclipse period, i.e., before new particles are detectable in prematurely lysed bacteria. Recombinants appear to arise at random at late stages of replication. A theory for the mechanism of recombination, taking into consideration various allelic linkages, has been elaborated by Visconti & Delbrück (100). The fact that no specific virus-like activity can be assigned to the "genetic" material is compatible with the reverse observation of Hershey & Chase (54) that the DNA of phage T2, "injected" into the infected bacterium, is not specifically identifiable. For the display of its specific viral propensities, the genetic material of phage T2 requires the vector of its protein membrane which is responsible for adsportion on host cells and for antigenic specificity (see below).

Animal viruses.—Because nothing is known about the possible independent functions of "genetic" and "nongenetic" materials of animal viruses, it is difficult to assess the significance of "genetic" experiments in relation to the problem of viral development. In the case of the Berry-Dedrick phenomenon, it has been amply shown [Smith (101)] that a deproteinized heated extract of rabbit myxoma mixed with rabbit fibroma virus will "transform" the latter into myxoma virus. It is not known, however, whether this process is a result of independent activity of the transforming factor in the infected host cell. On the basis of published evidence, one could even imagine that the change might occur *in vitro* before inoculation of the mixture.

Exchange of strain characters among type A strains of influenza virus has been reported by Burnet *et al.* (102 to 105) and by Appleby (106). The results were interpreted by Burnet, Fraser & Lind (105) in the sense of allelic interchange closely analogous to genetic recombination between bacteriophages. Some of the characters used by these authors as "genetic markers" may not be as reliable as seems desirable for this sort of work. Moreover, the stability of the "recombinants" described by them has not been established by serial passage. Hirst & Gotlieb (107), using the specific antigenic structure as markers, have been able to develop hybrid strains containing antigenic components from both parent strains. It was their experience that these forms usually were quite unstable and, on infecting the cell, dissociated into parent types. In addition, however, they have isolated a hybrid strain which does not dissociate on passage. Further elucidation

of these phenomena and also of the mechanism of multiplicity reactivation of ultraviolet-irradiated or heated influenza virus [Henle & Liu (108)] will undoubtedly contribute a great deal to our understanding of viral development.

Intermediate Forms: Specific Biological Properties Other than Infectivity and Their Relation to Viral Development

If, as is postulated for bacteriophage T2 reproduction (see above), the genetic material of the virus multiplies independently, it remains to account for the origin of other viral materials. Biochemical and genetic evidence suggests that infecting phage particles are capable of usurping the proper functions of the host cell to the advantage of their own species. It is, therefore, not too far-fetched to speculate on the possibility of synthesis of nongenetic components being under the control of viral nuclear material. This could be envisaged as analogous to the effect of transforming principle (i.e., DNA) on the configuration of a nongenetic substance (i.e., capsular polysaccharide) produced by pneumococci.

This suggests an approach to the problem of viral development which has been injected into discussions more and more, as different biological activities other than reproducibility have been recognized for many viruses. In this section, such activities will be enumerated, and their possible connection with viral development will be examined.

Bacterial viruses.—Herriott (109) has shown that T2 phage particles subjected to osmotic shock [Anderson (110)] release into solution their DNA, and that the resulting membranes ("ghosts") contain protein but no nucleic acid. These findings have been extended by Hershey & Chase (54) with S^{35}- and P^{32}-labelled T2. The ghosts contain phage-specific antigens, are adsorbed on sensitive bacteria, and kill cells on which they are adsorbed. An average of nine ghosts per bacterium is sufficient to reduce the turbidity of a bacterial suspension to less than 50 per cent in 15 min. at 37°C. [Herriott (109)].

From work by Doermann (58) and Anderson & Doermann (59) on premature lysis of infected *E. coli* by any of several methods (KCN, T6 lysis, sonic vibration), it has become clear that infectious virus increases during the entire second half of the latent period. Levinthal & Fisher (111), using the "decompression method" of Fraser (112) to induce premature lysis of T2-infected *E. coli*, showed that the appearance of new mature phage particles was preceded by that of membranous structures which were visible under the electron microscope as "doughnuts." Noninfectious "doughnuts" eventually acquire tails but lack the electron scattering power of fully developed particles. The similarity of these "doughnuts" to Herriott's osmotically shocked ghosts was pointed out. However, DeMars *et al.* (113) subsequently showed important differences. "Doughnuts" liberated by cells after proflavine treatment contain $\frac{3}{4}$ of the sulphur, but only $\frac{1}{6}$ of the phosphate of mature phage particles. They are not adsorbed on *E. coli*

and do not kill bacteria. They contain a complement-fixing antigen but, in contrast to plasmolysed ghosts, do not absorb phage-neutralizing antibody. DeMars *et al.* interpret the low P content of the "doughnuts" to mean either that nucleic acid and protein moieties are formed separately and merge at maturation or that, in prematurely liberated particles, the two fractions separate.

In addition to this incomplete viral particulate, DeMars *et al.* (113) found in T2- or T6-infected *E. coli* an "ultrafiltrable" (i.e., passing filters of 50 mμ average pore diameter) antigen which blocks the phage-neutralizing power of serum. This antigen does not fix complement. It is apparently identical with similar substances described long ago by Burnet (114). Its increase coincides with or slightly precedes that of active phage [Luria (97)]. The "soluble" complement-fixing antigen described by Rountree (115) as arising during the latent period in T5-infected *E. coli* would not be in line with these observations, and Hershey & Chase (54) suggest that it might be related to persistence of antigenic material of the infecting phage particles on bacterial debris. (It should be noted, however, that the findings of Hershey & Chase were made on T2 and may not be equally valid for T5.)

Animal viruses.—Although various biological activities other than infectivity are associated with many viruses or specific products of host-virus interaction, the relation of these activities to viral development has been studied in detail only in a few instances. Activities under consideration are "toxicity," viral hemagglutinins, viral antigens, and the "stagewise" development of influenza virus.

Animal viruses: "toxicity."—The "endotoxins" of viruses of the lymphogranuloma venereum-meningopneumonitis group of viruses can be separated from the virus particles [Rake & Jones (116)], but there is no evidence for their independent production in the course of viral development. The "toxic" properties of other viruses [influenza (117, 118), Newcastle disease (119, 120), and equine encephalomyelitis (121)] have invariably been proportional to their infectivity and no distinct toxic factors have been isolated from the viral particles or from infected tissues.

Animal viruses: viral hemagglutinins.—Some of the viral hemagglutinins (HA) have physical properties different from those of the infectious particles and might therefore be thought of as intermediate viral forms, breakdown products, or reaction products of the host-virus complex. This is particularly true for the HA^2 of the pox viruses which have been likened by Burnet (122) to phospho-lipid-protein complexes in the nature of soluble antigens and which Chu (123) believes to be a virus-host interaction product. Gillen *et al.* (124) differentiate a "soluble" and a virus-bound HA with different properties, a view questioned by Briody (125). Briody & Stannard (79) were unable to show independent production of HA and infective virus.

For most of the neurotropic viruses, intimate association of HA with infective particles has been demonstrated. Findings of Morris (126) suggest the possible existence of a "soluble" HA of Theiler's GDVII virus, but its

production in infected mouse brain closely parallels that of infectious virus.

In the case of pneumonitis virus of mice Ginsberg & Horsfall (81) have shown evidence for the (enzymatic?) dissociation of HA and infectious virus during the "eclipse" period, but in the incremental phase the two properties showed parallel rises.

Granoff *et al.* (127) have described the presence in Newcastle disease-infected allantoic membranes of a noninfectious HA. The corresponding allantoic fluids contain little, if any, of this material. The noninfectious HA lacks the hemolytic property of the infectious HA. It also differs from the latter in other features of the red cell-virus interaction (128). Thus far, it has not been possible to show (127a) that this noninfectious HA is produced in the membrane at a rate different from that at which infectious virus increases.

The relation of HA of influenza viruses and fowl plague virus to viral development has been studied in greater detail and will be reviewed in a moment.

Animal viruses: viral antigens.—The natural occurrence of antigenic materials which are not associated with the infectious viral particles seems to be a feature of almost all animal virus infections. Examples of "soluble" complement-fixing (C-F) antigens are numerous and need not be listed here [see Smadel (129)]. It is very likely that even many of those antigens not usually referred to as soluble are, in fact, distinct from the infective particles; e.g., antigens of the various encephalitis viruses, of yellow fever and dengue remain undiminished in the supernatants at centrifugal speeds which sediment more than 99 per cent of the infectious virus [Casals (129a) Schlesinger (129b)]. However, because of the relative insensitivity of serological tests, it is usually not possible to relate the development of these antigens to that of infectious virus. It has been noted in many instances that the soluble antigens are less specific than antigens associated with the virus proper, and this suggests that they may be interaction products of virus with host cell material derived from the cellular "matrix" [Fulton (130)].

"Dissociation" between the development of antigen and infective virus has been reported for herpes simplex. Jawetz *et al.* (131) showed that a soluble, skin-reactive antigen appeared in herpes-infected eggs two days before an increase in infective virus was measurable. This situation seems to be basically different from the demonstration by Salk & Bennett (132) that a strain of Lansing poliomyelitis virus of low mouse-pathogenicity multiplies in mouse brain to the same antigenic titer as do mouse-adapted strains. In this case, there is no evidence for distinct development of antigen and virus. Rather, the antigen titration in this system fulfills the same function as the use of sensitive indicator hosts does in others [cf. differential adaptation of dengue virus to suckling and adult mice, Schlesinger & Frankel (133)].

Animal viruses: the "stagewise" development of influenza virus.—The concept of stagewise development of influenza virus in the chorioallantoic membrane is based on the finding that, after intra-allantoic inoculation of large doses of virus, an increase in soluble C-F antigen[2] [Hoyle (66, 67, 69)],

V antigen and hemagglutinin [Henle *et al.* (68, 70)] precedes a rise in infectious titer. On the interpretation of various details and their role in relation to the problem of viral development, there is room for disagreement.

Hoyle believes that on entry into the cell, the viral elementary body disintegrates, releasing smaller units (S antigen and HA) from a lipid membrane. According to him, the basic replicating unit is the S antigen which is ribonucleoprotein in nature (134). An increase in S antigen is said to precede that of V antigen or HA, and the latter is said to be formed from the S antigen, its specificity being patterned after that of the infecting agglutinin. The complex formed by S antigen and HA is enzymically active, and combines with a lipid envelope which is derived from the cell wall.

The basis of Hoyle's imaginative view lies in some observations of his on the disintegration of viral elementary bodies under treatment with ether (134), which is said to release from a lipid membrane smaller S antigen and HA units. Electron micrographs of representative particles have been published by Hoyle, Reed & Astbury (135). General acceptance of Hoyle's findings, especially of his chemical characterization of the lipid membrane and of the S ribonucleoprotein, will depend on more complete documentation or independent corroboration of his data. Even if disintegration with ether yields fractions of these diverse activities, these need not be identical with the physical bearers of similar activities as they arise in the course of intracellular development (cf. the similarities and differences between plasmolyzed membranes and intracellular "doughnuts" in the case of T2), just as virus inactivated by other artificial means is not necessarily identical with a naturally arising noninfectious form.

Wiener, Henle & Henle (136) were able to remove S from viral antigen by sonic vibration which leaves infective and HA titers undiminished. Kirber & Henle (137), along with Fulton (130), look upon S antigen as a "matrix" substance, i.e., a product of virus-host cell interaction, rather than an independent replicating unit. Liu & Henle (70) found concurrent rise in S antigen and HA to precede rise in infectivity. This divergence from Hoyle's observation may be apparent only because test methods of different sensitivity are involved.

The critical element in these observations seems to rest in the fact that they can be made only in eggs inoculated with large doses of infectious virus. When smaller inocula are used, the amounts of HA and C-F antigen produced during the initial growth cycle are too small for detection. On the other hand, Henle & Henle (138) and von Magnus (139 to 142) have shown that in eggs infected with large doses the yield of infective virus is lower than after infection with dilute inocula, although HA and antigens rise to maximal levels. Serial passage of undiluted allantoic fluids leads to an increasingly higher proportion in the yield of noninfective HA, until about the third or fourth passage when a minimal EID/HA ratio[2] but also a minimal total viral yield is reached. On continued serial passage, the EID/HA ratios of yields fluctuate more irregularly (140, 143).

The noninfectious HA obtained by von Magnus' procedure has a lower sedimentation rate ($S_{20} = 400$) than fully infectious "standard" virus ($S_{20} = 700$) [Gard *et al.* (144)]. Electron micrographs of such particles obtained from allantoic membranes and adsorbed on laked erythrocytes reveal flat, inhomogeneous membrane-like structures with rough surface architecture and with average diameters larger than those of homogeneous, smooth, spherical "standard" particles (see page 103).

A plausible explanation of these observations, and one which the reviewer has adopted in the past (85, 145), is that offered by von Magnus. According to his view, large doses of virus infect simultaneously all or nearly all susceptible cells. The ratio of noninfectious, "incomplete" virus to fully infectious virus in the yield depends on the proportion of the two in the seed. A cell infected with infectious virus will only produce infectious virus. The noninfectious form does not multiply. In cells infected with a mixture, multiplication occurs, but the incomplete virus interferes with the maturation of newly produced particles. It is assumed, then, that "standard" virus also contains a certain proportion of "incomplete" virus (142). One flaw in this concept is the irregular, unpredictable result of passages carried beyond the one giving the highest proportion of "incomplete" virus. The second objection is that interference of this type, i.e., interference with the final stage of viral development only, has not been demonstrated with homologous ultraviolet-irradiated virus even if it is administered at the time when the "maturation" is presumably occurring. The concept of "autointerference" involved here is based on the idea of stagewise development discussed above, but the latter in turn leans on von Magnus' experiments for support.

Some clarification may come from studies on the fate of influenza virus in mouse brain (85). Significant "autointerference" does not occur when even large doses of the neurotropic variant of the WS strain (WS-N) are inoculated intracerebrally. On the other hand, nonneurotropic strains (PR8, WS, Lee) induce in mouse brain a single cycle of viral multiplication during which C-F antigen and HA increase without commensurate rise in infectious titer.[4] The latter either decreases progressively or stays at a constant level (WS strain only) for several days. Within a fairly narrow range of seed inocula (about 10^5 to 10^8 EID), limited by the relatively low sensitivity of C-F and HA titrations, the yield of these two activities is proportional to the input. After inoculation of smaller doses of virus, we have not been able to show an increase in any activity except, again, occasional minor upward fluctuations in the infectious titer of WS (85, 143). Cairns has confirmed these findings in essence, especially for the Lee strain (146). He reported, however, that inoculation of PR8 in relatively small doses led to a single-step increase in infectious titer (147), a finding which suggests minor differences between Cairns' and our lines of PR8. The important thing is that

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[4] The electron microscopic characteristics of noninfectious HA from mouse brain will be discussed below (p. 103).

here is an organ in which at least one strain of influenza virus (WS-N) can reproduce true to type unimpeded by "autointerference," while the multiplication of others is restricted to a single cycle and to the production of only or predominantly noninfectious virus. These and other considerations leave open the possibility that the "noninfectious" virus may arise in mouse brain as a result of selective multiplication of an avirulent variant rather than of "incomplete" development. A similar alternative might be left open for the von Magnus phenomenon in eggs, especially if it is conceded that even the initial "standard" inoculum is inhomogeneous (142). It is conceivable that under conditions of massive infection an "avirulent" variant establishes dominance over a virulent one, as has been postulated in the case of *Salmonella* phages (2). This approach to the problem might also reconcile the interpretations by von Magnus and those by Cairns & Edney (148) who calculated that production of "incomplete" virus occurs even when eggs are infected with an average of only 0.01 viral particles per cell. Their calculations led them to postulate that production of "incomplete" virus is attributable to shortage of some factor responsible for conversion into "complete" virus, and that this shortage develops before and independently of depletion of susceptible cells.

The reviewer takes the dogmatic position that noninfectious HA cannot be considered as a proved developmental stage of influenza virus unless it can be shown in some way that all newly formed viral particles have to go through this stage before they become infectious. The same reservations apply to similar findings on fowl plague virus reported by Schäfer and co-workers (76, 149).

Plant viruses.—Markham (150), working with turnip yellow mosaic virus, has separated a noninfectious, nucleic acid-free fraction from an infectious fraction which is exceptionally high in NA content. Markham does not believe that these two fractions represent different developmental stages of the virus.

CONTRIBUTIONS OF THE HOST CELL TO VIRAL DEVELOPMENT

Host-specific antigens have been found incorporated into, or firmly associated with, a number of viruses [e.g., Rous sarcoma (151); influenza (152, 153); equine encephalomyelitis (154); fowl plague (155)]. The studies of Smith (156) suggest the possibility that host-specific antigens might be inseparably incorporated into influenza virus since it is only after heat denaturation that their presence can be unmasked. On the other hand, purified insect viruses [Bergold & Friedrich-Freksa (157)], bacteriophages [Cohen & Arbogast (158)], and plant viruses have been found to be free of detectable host-specific antigen.

In the system studied by Zinder & Lederberg (159), a bacterial virus serves as carrier of host-specific genetic material from one host species to another. While it is not assured that this "transduction" is more than passive transfer of material attached to the surface of phage particles by

chance, the mode of entry of phage-DNA, as described by Hershey & Chase (54), makes it easier to visualize chromosomal linkage between phage replication and host-specific determinants. Further elucidation of this phenomenon can be expected to yield information of most fundamental importance.

PHYSICAL ASPECTS OF VIRAL DEVELOPMENT

In the previous sections, emphasis was placed on biological tools in the recognition of developmental stages. Where physical data are available to characterize biologically active phases of viral activity, they have been mentioned. There is, in addition, a wealth of information on the purely physical aspects of viruses or virus-infected cells in which correlation with biological observation is lacking or left in the realm of speculation. The "resting" forms of many viruses, examined under the electron microscope, show a great deal of heterogenicity and, in some instances, this has been interpreted as representing viral particles at different stages of development. In view of the crudeness of many bio-assay methods, this interpretation often seems to be highly premature. This applies equally to claims for the demonstration of "developmental stages" inside infected cells.

PHYSICAL CHARACTERIZATION OF "INTERMEDIATE" FORMS OF VIRAL DEVELOPMENT

Bacterial viruses.—The formation and characteristics of phage "doughnuts" of *E. coli* phage T2 (111) have already been discussed. It is tempting to view these nucleic acid-free "membranes" as established precursors of the final virus particles. Herčik (160) saw particles of different degrees of electron scattering power in T2-infected bacteria. Although he believed this to be a result of greater hydration of mature particles, it would seem l kely that the less dense particles he described were the "doughnuts" of Levinthal & Fisher (111).

Wyckoff (161) described the presence of filaments in *E. coli* infected with T2 or T4. These filaments were sometimes segmented into subunits of a size corresponding to the mature virus particles.

Beutner *et al.* (162) and Mudd *et al.* (163) have recently summarized light and electron microscope data on the intracellular development of phages. Their own beautiful photo and electron-micrographs of T2-infected *E. coli* show convincingly that recognizable phage particles do not appear until 15 minutes after infection, i.e., during the second half of the latent period. The first phage-like particulates are tailless and appear to be identical with the earlier mentioned "doughnuts." These findings confirm and extend previous observations by Luria & Human (164), Murray *et al.* (165), and those by Levinthal & Fisher (111). The electron microscopic demonstration of recognizable phage particles during the second half of the latent period is in good agreement with the results of intracellular phage assays obtained by means of premature lysis [Doermann (57, 58); Anderson & Doermann (59); Kay (60); Joklik (61)].

The use of a more indirect physical method, that of comparing ultra-violet or x-ray sensitivity of free phages and of phage-bacterium complexes [Luria & Latarjet (166); Latarjet (167); Benzer (168)], has yielded valuable information corroborating present concepts on phage development. When T2 enters *E. coli*, no essential change in radiosensitivity from that of free phage occurs during the first seven minutes, suggesting that the genetic phage material has remained qualitatively unaltered. Rapid reduction in radio-sensitivity between the seventh and ninth minute point to this interval as the critical period in which the average effective cross section of phage precursors is reduced (168). This would coincide with the beginning of DNA synthesis [Cohen (169)]. From the ninth to the twelfth minute, the slope of inactivation curves changes rapidly from one-hit to multiple-hit character, and this is taken to indicate that actual replication is restricted to this short time span (167). From the thirteenth minute on until lysis progressive maturation of phage manifests itself in gradual return of the slope to that characteristic for free phage.

A somewhat simpler picture emerges from similar studies on the smaller phage T7 which in its free state appears to have no membrane or tail [Benzer (168)]. Here, there are no qualitative changes in the slope of inacti-vation curves throughout the growth cycle, but only a progressive shift in the direction of multiplicity. In this case, multiple viral units are detectable as radiation targets before they become detectable as infectious units by Doermann's technique. All of this is compatible with the concept of inde-pendent replication of the phage-genetic material preceding maturation processes [Luria (97)].

Animal viruses.—It has been pointed out above that the significance of "soluble" antigens or small HA units in relation to the development of a number of viruses is in doubt, because the actual conversion of these phases into infectious viral particles has not been demonstrated unequivocally.

Animal viruses: filamentous forms.—It was shown first by Mosley & Wyckoff (170), later by Heinmets (171), Chu, Dawson & Elford (172), and Dawson & Elford (173), that preparations of influenza virus contained not only spherical but also filamentous particles. The latter have the same di-ameter as spherical particles and are often segmented into spherical units. The proportion of one or the other type of particle varies with different strains of influenza virus, recently isolated strains being particularly rich in filaments (172). Similar filamentous forms have been seen in preparations of fowl plague [Dawson & Elford (173); Flewett & Challice (174)] and New-castle disease virus [Bang (175)]. Filaments are adsorbed on agglutinable red cells along with spheres (171, 173) and are believed to represent active viral particles. Their emergence from influenza or Newcastle disease virus-infected cells has been studied electron microscopically by Wyckoff (176) and Murphy & Bang (177). Filaments are now generally looked upon as representing a "developmental stage" of these viruses. It is believed that they may be formed from cytoplasmic protrusions and that the segmenta-

tion occurs preliminary to their breaking up into spherical viral units [Hoyle (67)]. Murphy & Bang (177) have called attention to the distinguishing features of similar protrusions seen on occasional normal cells. The precise mechanism of the release of filaments from cells is not known. The idea that virus is liberated by the separation of such cytoplasmic protrusions from the cell mass would be in line with the absence of detectable changes in the cell structure at the time of virus release (177). A similar explanation has been offered by Robinow (178) for the "stalked" forms of vaccinia virus. Whether there exists any difference in biological activity between spherical and filamentous forms, cannot be decided on the basis of available information.

The situation seems to be somewhat different in the case of fowl plague virus. Here, it has been shown by Flewett & Challice (174) that the nucleus and especially the nucleolus, undergo marked changes in early stages of infection. Intranuclear inclusions consisting of highly refractile material replace the nucleoli, finally break up into numerous small granules which eventually fill the entire nucleus. While these nuclear changes occur, the cytoplasm undergoes vacuolization and mitochondria disappear. Filaments are seen streaming from the disintegrating nucleus into the cytoplasm. The authors believe these to be viral filaments which will segment into elementary bodies.

Animal viruses: morphology of "incomplete" forms of influenza and other viruses.—The question of whether or not noninfectious hemagglutinins arising under certain conditions in eggs or mouse brain infected with influenza viruses represent developmentally incomplete forms, has already been examined. Such forms, obtained by von Magnus by serial egg-to-egg passage of undiluted infected allantoic fluids (140), have a sedimentation constant of about 400 S as compared with 700 S of the "mature" infectious particle. Schlesinger & Werner (179) studied "incomplete" virus particles obtained from mouse brain (see p. 99) under the electron microscope. Such particles, adsorbed on laked fowl erythrocytes show far more inhomogeneity in size and shape than ordinary influenza virus. In shadowed preparations, most of the particles appear as flat, bag-like, or doughnut-shaped bodies with average diameters far greater than those of mature particles. The surface of these particles is rough, suggesting aggregates of smaller internal structures. This is borne out by unshadowed preparations which reveal clusters of minute granules sometimes surrounded by areas of intermediate electron density. "Incomplete" virus particles from allantoic membranes produced in eggs according to von Magnus' method look similar to the mouse brain particles but have not yet been examined without shadow-casting.

The morphological differences between these "incomplete" particles from either source and "mature" infectious particles from allantoic fluid or membranes is very striking, but here, again, it must be stressed that it has not yet been shown that under ordinary conditions each developing viral particle would go through a comparable phase.

The appearance of the "incomplete" influenza virus particles is reminiscent of the "sub-virus" granules and granular particles sedimented with cellular debris from tissue infected with molluscum contagiosum virus [Rake & Blank (180)]. Further studies on the intracellular development of this agent have recently been published by Banfield *et al.* (181). These authors suggest that viral particles develop in association with (or from) a cytoplasmic trabecular "matrix" by process of segmentation into pro-virus units which are larger than the final, brick-shaped particle, and mature by condensation. Vital staining of sections of molluscum lesions carried out by Rake & Blank suggests that viral DNA arises by transformation of cytoplasmic RNA[2] which seems to form the trabecules in which Banfield *et al.* find the elementary bodies forming. The latter authors also observed that sectioning of molluscum virus particles left hollow, ring-like membranes suggesting existence of a formed cortex and a less dense interior.

Similar structures have been reported on by Gaylord *et al.* (182, 183) for vaccinia virus. To what extent the variable appearance of particles described by them reflects definitive stages in viral development is conjectural.

Plant and insect viruses.—Turnip yellow mosaic virus is homogeneous by the criteria of electrophoresis and crystallization, but on ultracentrifugation dissociates into two components with sharply separated sedimentation boundaries (150). The "top" fraction (20 to 30 per cent) is protein which is free of NA,[2] has low antigenicity, and is noninfectious. The bottom portion contains NA in high concentration and is infectious. Markham cites a number of reasons which make it unlikely that the NA-free fraction represents a developmental stage of the virus. He postulates that the host cell's mechanism for NA synthesis may be unable to keep pace with its ability to synthesize viral protein.

Polyhedral and capsular virus diseases of insects have been profitable objects of physical and physico-chemical study. Bergold and his associates have presented numerous excellent electron micrographs of forms which they believe to represent different developmental stages of viruses. This work cannot be described here in detail and should be consulted in the original publications [References in (9)].

VIRAL DEVELOPMENT AND CELLULAR PARTICULATES

An additional key to viral development may be found in the quest for association of viral activity with various components of infected cells. This approach, based on microscopy or on cytological techniques recently reviewed by Schneider & Hogeboom (184), has been applied to a variety of virus-host complexes.

Bacterial viruses.—Infection of *E. coli* with T phages leads to disintegration of the cellular nuclear material, intensity of which varies for different strains [Luria & Human (164)]. Chromatinic granules appear in the cytoplasm at first close to the membrane then gradually filling the entire cell body (163, 164, 165). According to Mudd *et al.* (163), mitochrondria are not

affected by phage infection. Hershey & Chase (54) suggest that DNA of phage T2, upon entering the cell, becomes integrated into a (preformed?) intracellular structure. Its nature is not as yet clearly defined cytologically.

Animal viruses.—Studies by Francis & Kurtz (185) on herpes simplex virus suggest that the intranuclear inclusions seen in infected cells are not associated with virus particles. Ackermann & Kurtz (186) have presented evidence which they interpret as revealing close association of herpes virus with mitochondria. They found approximately 80 per cent of the virus "free" in the cytoplasm and about 16 per cent intimately tied to the mitochondria, and assume that multiplication takes place in association with the latter, with virus released into the cytoplasm after their deterioration. Similar suggestions may be inferred from work on rabies-infected mouse brain reported by Habel *et al.* (187). The relation of poliomyelitis and of mouse encephalo-myelitis (FA strain) viruses to cellular constituents has been investigated by Schwerdt & Pardee (188), Gard & Östlund (189), and by Kaplan & Melnick (190). All agree that most of the virus is present in the "cyto-plasmic" fraction of mouse brain homogenates. Gard & Östlund extracted about 90 per cent of the "nuclear" virus with hypertonic NaCl solution or with ficin and concluded that the virus is probably not firmly included in the nuclear mass. Kaplan & Melnick take the opposing view, and consider the virus present in the "nuclei" as related to its development. This interpretation is supported by the finding that the "nuclei" of paralyzed mice contain far more virus than those of nonparalyzed ones sacrificed at the same time. One possible reservation about this approach may be that the "cyto-plasmic" fraction obtained from an organ like mouse brain contains not only the neuronal cytoplasm but also that of other cellular elements as well as extracellular virus. The "nuclear" fraction likewise contains presumably elements from all types of cells, and the predominating type of cell in a brain in which neuronal destruction and infiltration with glial and hematogenous cells has advanced may well differ from one in which such changes are minimal.

A critical evaluation of the significance of cellular particulates in relation to viruses has recently been presented by Porter & Kallman (191).

CHEMICAL ASPECTS OF VIRAL DEVELOPMENT

CHEMICAL CHARACTERIZATION OF DISTINCT BIOLOGICAL FUNCTIONS

Certain observations germane to this section have already been reviewed in their proper context above and need not be gone into in detail. The most far-reaching of these is the demonstrated separation of "genetic" nucleic acid from "nongenetic" protein moieties in the case of phage T2 (54), and the suggestion of a similar situation in the case of turnip yellow mosaic virus (150). Although chemical characterization of the amino acids and DNA bases of insect viruses and of polyhedral protein has gone far, the relation of one to the other in development remains to be studied (9).

Viral Development and Host Cell Metabolism

Bacterial viruses.—The metabolic aspects of phage multiplication have been covered in several recent papers (50, 192, 193), and a recapitulation of the vast amount of data is impossible within the limits set for this paper. To those, who approach the problem of virus infection primarily from the biological point of view, one of the most exciting aspects of the biochemistry of phage-infected cells is the degree to which developing phage has been shown to take over control of metabolic activities at the expense of the host cell's normal functions. This has been elegantly confirmed in the most recent work on the difference in metabolic response between bacteria infected with "temperate" and with "virulent" phage strains. When *P. aeruginosa* is infected with lytic phage ("virulent"), and the amount of glucose as source of C in the medium reduced even to low levels, this fails to stop phage production, although it may prolong the latent period. In cells infected with temperate virus (i.e., in induced lysogenic cells), glucose can be reduced to levels at which bacterial growth is suppressed, and yet phage production proceeds [Jacob (194)]. Siminovitch & Rapkine (195) have analyzed the biochemical modifications in lysogenic *B. megatherium* after induction. Infection with "virulent" phages leads to arrest of bacterial growth and of synthesis of RNA or adaptive enzymes, and oxygen uptake remains at the level at which it was at the time of infection. "Temperate" phage has none of these effects either in induced host cells or even in sensitive bacteria infected with phage originating from a lysogenic strain. This indicates that "temperate" virus interferes much less with the bacterial metabolism than its "virulent" counterpart. Siminovitch & Jacob (196) further showed that the biosynthesis of β-galactosidase by lysogenic *E. coli* K12 (λ) may be induced by addition of lactose to the medium even after ultraviolet irradiation has set in motion the development of phage. The later lactose is added to this system, the lower is the rate of adaptive enzyme production. Metabolic investigations by Jacob, Siminovitch & Wollman (25) have also thrown additional light on the striking similarities between lysogenic and colicinogenic *E. coli*. As has already been mentioned, liberation of colicine can be induced, like that of phage in lysogenic strains, by ultraviolet-irradiation, mutagens, or carcinogens. Cells so induced continue to grow, to synthesize respiratory and adaptive enzymes, RNA, and DNA. In all these respects, except with regard to uninterrupted DNA synthesis, such induced colicinogenic cells behave like induced lysogenic cells. In contrast, exogenous colicine behaves like exogenous "virulent" phage: it kills sensitive bacteria and stops all other synthetic activities. The main difference is that colicine does not reproduce.

Other viruses.—The high degree of accuracy with which specific metabolic effects can be measured in phage-bacterium systems, has not been achieved for other virus-host complexes. Many differences between normal and infected organs or tissues have been reported, but in most instances, the infection had already progressed to a point where cells were destroyed or damaged.

Therefore, it is difficult to distinguish changes related to viral development from secondary changes, even if observed effects appear to be selective [e.g., selective inhibition or stimulation of amino acid incorporation by minced baby mouse brain infected with GDVII virus reported recently by Moldave *et al.* (197)]. Similarly, it would be premature to infer from the virus-inhibiting activity of various antimetabolites that specifically blocked metabolic steps can be identified as directly involved in conversion of one developmental stage into another.

CONCLUDING REMARKS

Studies on various properties of the extracellular "resting" forms of many viruses have made big strides, driven in part by the thought that physical and chemical characterization of these forms might give clues to the nature of viruses and to their ultimate control. This thought stems quite naturally from the fact that, from the practical point of view, the resting form is the concrete phase which acts as a parasitic organism and is capable of inducing disease.

A more general biological approach to viruses has shifted emphasis to other and more elusive phases in their ecology. It has led to fundamental revelations concerning the unique relationship between viruses and their host cells. Of these, only a few could be covered within the limits set for this review, and the selection had to be made arbitrarily. The principal conclusion drawn from the data discussed above is that exogenous origin, pathogenicity, and even ability to multiply may be irrelevant and fortuitous from the point of view of the virus. The mechanism by which a resting, extracellular entity is integrated into the cell and transformed into a kinetic, actively vegetating element or into an inactive, symbiotic pro-virus is apt to furnish the key to the characterization of viruses as a distinct biological class.

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NUTRITION OF MICROORGANISMS[1,2]

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The year 1952 saw research on microbial nutrition continuing at a high level of activity. The rich "mother lode" of new growth factors for lactic acid bacteria and related organisms has perhaps begun to fade, but any loss in glamour from this quarter has been offset, at least in part, by many careful investigations that were designed to expand our knowledge of nutritional and metabolic interrelationships, with respect both to biocatalyst systems and to those nutrients that form the structural units of protoplasm. Direct growth studies have been supplemented to an increasing degree with experiments in resting cells and cell-free systems, and organisms of other biological phyla also have been employed with success.

Experiments that deal strictly with metabolic interrelationships are emphasized in the chapter on Metabolism of Microorganisms, and hence will receive relatively less attention here. Also reduced in discussion, or regrettably omitted because of space limitations, are many papers dealing with general growth characteristics of microorganisms, production of antibiotics and their influence on microbial growth, and the effects of pharmaceutical agents.

New Growth Factors

Lipoic Acid (Thioctic Acid; Pyruvate Oxidation Factor; Protogen; Acetate Replacement Factor).—The research on lipoic acid contrasts sharply with the implied slackening of activity in the discovery of new microbial growth factors; indeed, in several respects the developments in the chemistry and biochemistry of this metabolite are outstanding for the year 1952. In part, as a result of the improvements in isolation techniques during recent years, and, in part, because of widening experience with a variety of biological systems, progress in knowledge concerning lipoic acid has been most rapid. Two bacterial and one protozoal growth systems and one bacterial oxidative system were under simultaneous investigation in the four laboratories that had independently reported the existence of this factor.

[1] The survey of the literature pertaining to this review was concluded in January, 1953.

[2] The following abbreviations are used in this chapter: ATP (adenosinetriphosphate); B.R. (*Butyribacterium rettgeri*); CF (citrovorum factor); CoA (Coenzyme A); DMDA (1,2-dimethyl-4,5-diaminobenzene); DPN (diphosphopyridine nucleotide); GG (N-glucosylglycine); LBF *Lactobacillus bulgaricus* factor≡pantetheine); LT (lipothiamide); LTPP (lipothiamide pyrophosphate); PA (pantothenic acid); PAB (*p*-aminobenzoic acid); PAC (pantothenic acid conjugate); PGA (pteroylglutamic acid).

Previous reviews (1, 2) have pointed out the probable identity of protogen (3, 4, 5), the acetate replacement factor (6), the pyruvate oxidation factor (7, 8), and lipoic acid (9 to 12). In addition, the B.R.[2] factor (13) which is required by *Butyribacterium rettgeri* for growth on lactate as the fermentable carbon source appears to be identical with lipoic acid (14, 15). On the basis of its fat-solubility and adsorption characteristics, it was also thought similar to vitamin B_{13} (1, 13, 16), but the latter identity has not been confirmed experimentally.

Lipoic acid was first isolated in pure form from liver by Reed, *et al.* (9); the term "lipoic acid" was introduced at this point to describe its fat solubility. The combined forms, in which the factor occurs in tissues, are water-soluble (14). The acid or alkaline extraction procedures employed reduced the number of bound forms from five to two or three; of these, one form was converted to another more polar form during the isolation procedure (10). A similar conversion was observed by Patterson *et al.* (17) in their isolation of protogen; they designated the process, protogen A→protogen B. These appear to correspond to α- and β-lipoic acids, designated by Reed *et al.* (18).

Studies on the structure of the lipoic acids (17 to 23) indicate that they are a family of dithioöctanoic acids and their derivatives. The first synthesis of a pure compound with full lipoic acid activity was carried out by Bullock *et al.* (21), who prepared the cyclic 6,8-dithioöctanoic acid, which they called 6-thioctic acid. It was confirmed as being identical with protogen A, after earlier work (20) had suggested the 5,8-ring structure. Protogen B is probably the corresponding monosulfoxide (17 to 19).

Work with *Streptococcus faecalis* and *Escherichia coli* on the behavior of lipoic acid has revealed valuable information regarding conjugated forms. It was found (24 to 27) that "lipothiamide" (LT) and "lipothiamide pyrophosphate" (LTPP) are active growth promoters in a mutant of *E. coli*. These products possessed R_F values during paper chromatography that were identical with those of two of the forms present in natural materials. The test system used was a strain that would not grow on either lipoic acid or thiamine, alone or in combination, but which grew luxuriantly on LT[2] or LTPP[2] as well as the naturally occurring forms of lipoic acid. Soluble enzyme preparations from this mutant were activated by LTPP (but not by thiamine, lipoic acid, or LT) to catalyze the oxidation of α-ketoglutarate or the anaerobic dismutation of pyruvate. These reactions thus reveal a definite role for the lipoic acid system in the oxidative decarboxylation of pyruvate and α-ketoglutarate, and thus explain its need by *S. faecalis* as the pyruvate oxidation factor, or by *Lactobacillus casei* as the acetate replacement factor, since acetate (as acetyl CoA) is an early product of pyruvate oxidation.

The question naturally arises whether diphosphothiamine may have separate functions from lipoic acid. This seems assured, since the simple decarboxylation of pyruvate to yield acetaldehyde requires only diphosphothiamine (28). Likewise, not all acetate forming systems require lipoic acid

(29). Thus the term "acetate-replacing factor" is somewhat misleading. It is also evident from this that LTPP is part of the oxidizing system rather than of the decarboxylating system alone. The suggestion has been made that pyruvate (or α-ketoglutarate) is oxidatively decarboxylated to form acetyl (or succinyl) LTPP, and the acyl groups are then transferred to CoA to give acyl CoA and regenerate LTPP.

Lipoic acid has not yet been shown to be a requirement for higher animals, and thus can not yet be properly included in the "vitamin" category. It occurs in animal pyruvic and α-ketoglutaric oxidases (30, 31), and there seems little doubt that it is an essential metabolite.

Coenzyme III.—Cell-free extracts of *Proteus vulgaris* were shown by Singer & Kearney (32, 33) to require an unknown factor for the oxidation of cysteine-sulfinic acid to cysteic acid. The factor was found in high concentration in yeast, and it was thought to be a possible participant in sulfur metabolism in this and other species of organisms. Isolation of the factor and structural studies revealed it to be a nucleotide, probably identical with nicotinamide-ribose-5-pyrophosphate, i.e., it lacked the adenosine structure found in coenzyme I. It was called coenzyme III because of its similarity in structure to the other pyridine nucleotides.

N-Glucosylglycine.—It has long been known (34) that heat activation of the culture medium is required to insure growth of many lactic acid bacteria. This activation has in general involved either (*a*) the removal of oxygen and the formation of reducing substances (35 to 38) or (*b*) interaction of carbohydrate with phosphate or the nitrogenous components of the medium (35, 39, 40). Autoclaved cystine has been found toxic to *Lactobacillus bifidus* (41). The formation of the toxic substance(s) can be prevented by autoclaving cystine together with a reducing sugar. The toxicity may also be counteracted by addition, after autoclaving, of cystine, thioglycollic or thiomalic acids, reductone (the enediol form of hydroxymethylglyoxal), or cystine plus ascorbic acid, but not of ascorbic acid alone. The toxic substance formed from cystine is apparently an oxidation product, which may be elementary sulfur (41, 42).

Recently, it has been found in this laboratory (43, 44) that products of heating of glucose with inorganic phosphate and amino acids stimulate the growth of *L. gayoni* during short (12 hr.) growth periods. Glycine was found to be consistently superior to other amino acids in this conversion, whereas alanine produced substances that were strongly inhibitory. Synthetic N-glucosylglycine (GG) ethyl ester was as active as equal weights of yeast extract in an unheated medium. High levels of GG[2] were inhibitory; however, when this compound was heated separately and then added to the medium, good growth was regularly obtained. Other species that responded to unheated GG were strains of *S. zymogenes*, *L. acidophilus*, and *Leuconostoc mesenteroides*. The relatively low activity of GG suggests that the growth stimulation is due to conversion products from this compound. However, the relative superiority of glycine over other amino acids in producing active

material, and the strong inhibition caused by alanine reaction products, suggest that the heat activation reaction is relatively specific and that one or at most a few factors may be involved.

Lyxoflavin.—L-Lyxoflavin is an isomer of riboflavin, differing in the configuration around C_4 of the pentose chain. Vitamin properties have been claimed for the compound (45 to 48), but these have been challenged by others (49, 50).

In microorganisms, L-lyxoflavin is both inhibitory and stimulatory, depending upon experimental conditions. Experiments with *L. casei* show it to be slightly active (46, 47, 50, 51) alone or in the presence of optimum levels of riboflavin, and in *L. lactis* the riboflavin activity of lyxoflavin reaches 16 per cent (52). At the same time, competitive inhibition between the two flavins has been observed in *L. casei* and *Leuconostoc mesenteroides* (52), with inhibition indices of 50 and 35 in the two organisms. Growth promoting activity could not be demonstrated in *L. lactis* and *L. casei* with the D-isomer, or with galactoflavin, D-araboflavin, and α-ribazole [found in vitamin B_{12} structure (52)]. Thus, the experiments with microorganisms apparently have not yet established a separate metabolic role for lyxoflavin in biological systems, and the question (45) of whether lyxoflavin may be a new member of the B complex remains unsettled. Many flavoprotein systems have been described, and it is possible that one or more of these may contain moieties other than D-ribose. Further experiments are in order to determine definitely the existence of lyxoflavin in natural materials (53, 54) and its participation in growth and metabolism.

Miscellaneous factors.—Crystalline biocytin (55) has been established, through synthesis and structural studies (56) as ϵ-N-biotinyl-L-lysine. The synthetic product is identical in all respects examined to the biocytin isolated from yeast (57).

Biocytin has the same activity as biotin in reversing homobiotin inhibition in *L. casei*, although at high levels biotin is more effective. Both the natural and synthetic products combine readily with avidin (58). Biocytin is equally or slightly less active than biotin for aspartic deaminase activity (58), and it seems questionable whether biocytin is the active biotin coenzyme of the deaminase system (59).

Yeast extract (Basamin) supplies a factor for the stimulation of the early growth of a strain of *S. faecalis* (strain AB). The factor existing in the yeast extract has been identified as uridine (60). Although the latter shows only half of the maximum growth response produced by the concentrate, surface active agents (such as Tween 80) act synergistically with uridine to produce a growth response equivalent to that obtained from yeast extract. Malt sprouts, corn steep, whole liver, trypsin, and wheat gluten also are active.

A red crystalline compound has been isolated from bacterial and fungal extracts, which is called "coprogen." It can replace dung extract for the growth of *Pilobolus* fungi at 5 m μg./ml. (61). Hemin is also active, but is only 1/1000 as effective as coprogen.

Several nonpathogenic *Neisseria* are reported to require putrescine in a chemically defined medium containing 18 amino acids, inorganic salts, biotin and glucose. Maximum growth was obtained at a concentration of 1 μg./ml. Spermidine, agmatine, and cadaverine may be substituted for putrescine (62, 63), with cadaverine being least active.

A few years ago it was found that *Corynebacterium* Q required oleic acid for its growth (64). More recently it has been shown (65) that the oleic acid can be replaced by *cis*- and *trans*-palmitoleic, *cis*- and *trans*-heptadec-9-enoic, *cis*- and *trans*-vaccenic acids, with approximate equivalence in growth promoting ability. *Trans*-myristoleic acid gives very slight growth, while *cis*- and *trans*-gadoleic, *cis*-myristoleic, and *cis*- and *trans*-octadec-4-enoic acids are inactive.

Many of the indigenous soil bacteria depend upon essential growth factors supplied by soil extract but not by yeast extract. The nutrilite effect of the soil extract can be replaced by vitamin B_{12} in certain bacteria. Other microorganisms still require some unidentified growth-promoting substances in soil. However, soil extract can be replaced by the culture-filtrate of a certain soil bacterium which evidently produces the unidentified factor(s). This factor(s) also occurs in liver extract but presumably is different from folinic acid and other known factors (66, 67).

Microbacterium flavum requires an unidentified factor(s) for optimal growth in a synthetic medium along with Mg^{++}. It occurs in many natural extracts and is apparently different from previously described growth factors, a long list of which were examined for possible identity. The factor is stable to HNO_2 or H_2O_2 treatment and is separated as an oil from aqueous solution with acetone (68).

A specific growth stimulatory material which reduces the lag phase of *Staphylococcus albus* has been concentrated about 850- to 1700-fold from bovine plasma. The isolated stimulant is a glycoprotein. Many other natural materials, such as beef heart infusion, yeast extract, bacterial broth filtrate, blood, and urine also contain the activity, but not a serum ultrafiltrate (69, 70). It is suggested (70) that a specific sulfur-containing peptide structure may be involved, which, however, is different from strepogenin (71).

PANTOTHENIC ACID

The bulk of the progress in pantothenic acid research during the past year has been aimed at the biochemical functions of coenzyme A (CoA). These are considered *in extenso* in the chapter on Metabolism of Microorganisms. Other studies, which deal with the vitamin and some of its conjugates, are presented here.

Acetobacter suboxydans was the first microorganism to be found responsive to bound forms of PA[2] (72); the conjugates PAC[2] and CoA[2] are both more active for this organism than is free PA (72, 73, 74). Pantetheine (LBF) is also more active than the free vitamin (75). In addition it was found that one of the fragments formed from CoA by digestion with potato

nucleotide pyrophosphatase is ten to twenty times more effective in supporting the growth of *A. suboxydans* than is the free vitamin (76). From an acid hydrolysate of CoA a product was also isolated (76) that produced the same microbial effect. It contained 1 mole of phosphate per mole of pantothenate and no adenine or ribose. Both enzymatic and acid hydrolysis products lost their *A. suboxydans* stimulatory activity after dephosphorylation with intestinal phosphatase. However, liver enzyme treatment, which is believed to cleave the mercaptoethylamide linkage, did not alter the microbial activity. Based on this and other studies, it was concluded that the *A. suboxydans* stimulatory factor is a phosphate ester of pantothenate. However, none of the synthetic derivatives [namely pantoyl phosphate (77), α-monophosphate, γ-monophosphate (78, 79, 80), cyclic monophosphate (81), and diphosphate (82) of pantothenic acid] prepared by the present authors and by Baddiley & Thain was able to support the growth of *A. suboxydans*. The possibility remained that the mercaptoethylamine moiety may have been incompletely removed during degradation of CoA (76), and a diphosphate of pantetheine (LBF) was therefore prepared (83). It was only slightly active for *A. suboxydans*, but the γ-monophosphate of LBF[2] (84) was active after heating with casein hydrolysate (75). Very recently (85) it was reported that the CoA degradation product (76) may contain sulfur, and it therefore appears that a bound form of PA, active for *A. suboxydans*, is a derivative of LBF. Still to be explained is the fact that the conjugate PAC stimulates *A. suboxydans* growth but is inactive for *L. bulgaricus* (86).

PA-deficient *A. suboxydans* cells have been found unable to effect the normal oxidations of glycerol, sorbitol, or glucose (87). Addition of CoA to these cells restored a large part of the glycerol oxidizing capacity. Failure to oxidize dihydroxyacetone appeared to be responsible for the poor performance of the deficient cells.

By growing *Bacterium linens* in the presence of individual B vitamins, it was found (88) that either PA or *p*-aminobenzoic acid (PAB) was required for growth, but not both. Biotin stimulated growth in the presence of PA or PAB,[2] but had only slight effect in the presence of both vitamins. Assays of the cells showed that either PA or PAB was synthesized in the presence of the other; biotin reduced the synthesis of each. Sulfanilamide inhibition of PAB-stimulated growth could be reversed by either PA or biotin. While these relationships are unclear, they recall earlier experiments on similar interrelationships among PA, biotin, and folic acid (89), and between PA and PAB (90). Further knowledge of the metabolic functions of each vitamin may uncover these interrelationships. Also remaining to be clarified is the observation (91) that when biotin and pantothenate are lacking, *Pasteurella pestis* requires a mixture of 20 amino acids for growth. This is in contrast to the requirement of only phenylalanine, valine, isoleucine, cysteine, methionine, alanine, leucine, serine, and threonine in the presence of these vitamins.

A soluble enzyme preparation for coupling β-alanine and pantoate has been isolated from *E. coli*. The reaction is similar to peptide synthesis and requires ATP[2] as an energy source and Mg^{++} or Mn^{++} as activators. No coenzyme requirement could be demonstrated. In the solubilized enzyme preparation the disappearance of pantothenate (presumably to the coenzyme form) could be prevented by an analogue, tolyl pantothenone (92). Studies with pantothenate auxotrophs indicate that salicylate interferes with pantoate synthesis. Salicylate, in concentrations where inhibition of wild type *E. coli* is reversed by pantoate or pantothenate, does not inhibit a pantoate auxotroph (93).

The keto-analogue of pantoic acid, α-keto-β,β-dimethyl-γ-butyrolactone, shows activity equal to or greater than that of pantoic acid in certain mutants of *E. coli*, either as a growth factor or as a reversal agent. It is almost inactive in another *E. coli* strain and in *A. suboxydans* (94).

It had been observed (94) that α-amino-β,β-dimethyl-γ-hydroxybutyric acid (pantonine) could replace pantoic acid in certain microbial inhibitions, and it was suggested that pantonine may be a precursor of pantoic acid. In order to determine whether the biosynthesis of PA occurs through the coupling of pantonine with β-alanine and subsequent deamination, pantonyl-β-alanine was synthesized (95). It showed less than 0.1 per cent of PA activity, but after its deamination with nitrous acid, the activity increased. In the reversal of cysteic and salicylic acid inhibitions in *E. coli*, the compound showed the same activity as that of β-alanine and pantonine, respectively. In *E. coli* mutants, pantonyl-β-alanine could replace pantonine, but not pantoic or pantothenic acids. Previously (96), it had been reported that pantonyl-β-alanine had no activity toward lactic acid bacteria. However, the compounds prepared in the two laboratories possessed different melting points and nitrogen contents, and the identity of the compounds thus remains to be determined.

CITROVORUM FACTOR

The biochemical role of the citrovorum factor, as a participant in the metabolism of "active formate," has been reviewed thoroughly elsewhere (97, 98), and hence will not be developed here.

Further studies on the structure of CF[2] have appeared. Although the structure of the naturally occurring factor is not yet known, it evidently is closely related to a synthetic material produced by reduction of folic acid in formic acid, or the reduction of N^{10}-formyl folic acid. This compound [synthetic citrovorum factor; folinic acid-SF; leucovorin (99 to 102)] has the structure 5-formyl-5,6,7,8-tetrahydropteroylglutamic acid. It resembles closely the purified naturally occurring factor (103) both spectrophotometrically and chromatographically (104). The crystalline compound isolated from liver, as well as concentrates obtained from spinach and urine, show an absorption maximum at 285 mμ and a minimum at about 258 mμ. Corresponding peaks for leucovorin were displayed at 285 and 245 mμ (103).

The isolated CF possesses about twice the potency of leucovorin for *Leuconostoc citrovorum* (105). Since leucovorin contains an additional asymmetric center at carbon 6 (see Fig. 1), it has two diastereoisomers, the dL and lL forms (106). lL-Leucovorin[3] has been separated as the pure calcium salt and shows twice the activity of the mixture of the diastereoisomers in the microbiological assay for *L. citrovorum* (106). dL-Leucovorin was not obtained in pure form; however, a crude fraction showed lower microbiological activity. The determination of the exact relationship between leucovorin and CF must await the demonstration of the structure of the latter.
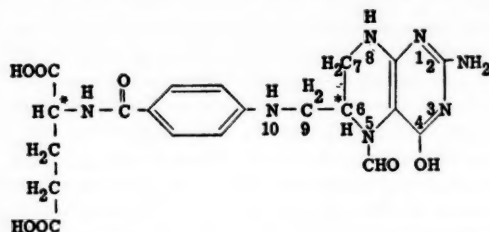
Fig. 1. Leucovorin (folinic acid - SF).

* = asymmetric carbon atoms.

Dried brewers' yeast contains the citrovorum factor, largely in a bound form that can be liberated by a hog kidney extract. Folic acid conjugase and the CF-liberating enzyme in hog kidney are very similar (107). Chick liver also contains a bound form of CF which can be set free by the enzyme in liver; the yields can be increased by adding ascorbic acid (108). The conjugated forms in yeast and liver samples are believed to be closely related or identical (109). "Extra" CF can be produced by enzymatic synthesis in liver slices, from pteroylglutamic acid (110), or from pteroyl di- or triglutamate (111).

An unidentified factor ("folacin substitute") in human blood with folacin-like activity for *L. casei* has been reported. It is different, however, from folacin, teropterin, or CF (112).

Still another form of the folacin-CF group has been reported to appear when hemopoietic tissue is incubated with folic acid. It is called hemofolin (113). The growth behavior of hemofolin in *L. citrovorum* is different from that of CF, in that treatment of hemofolin with hog kidney conjugase does not alter the type of response curve. It is perhaps not a glutamyl conjugate. However, it is similar to folinic acid in the reversal of aminopterin and of chloramphenicol inhibition in *L. citrovorum*. At certain levels of antibiotic

[3] "dL" and "lL" apparently refer to (+) and (−) forms of two diastereoisomers (9).

inhibition, hemofolin is much more active than folinic acid. No extensive isolation has been attempted due to the lack of suitable starting material. Judging from certain patterns in the reversal of inhibition, hemofolin is perhaps one of the metabolically active forms of folic acid.

Vitamin B_{12}

Several new compounds related to vitamin B_{12} were reported during the past year. From the broth of a rumen anaerobe, Pfiffner et al. (114) have isolated two series of vitamin B_{12} like substances in crystalline form, and a third series as amorphous powders. Each series has two compounds, namely the cyano- and the corresponding hydroxy-derivatives. The crystalline series are highly active as growth factors for certain lactobacilli but are inactive in animals. The crystalline compounds are called pseudovitamin B_{12} and pseudovitamin B_{12b}, or cyano-β-cobalamine and cyano-γ-cobalamine. The amorphous pair has no demonstrable biological activity. Pseudovitamin B_{12} differs from vitamin B_{12} in the occurrence of adenine instead of 5,6-dimethylbenzimidazole in the nucleotide portion of the molecule (115). Both cyano-β- and γ-cobalamine have been tested as growth factors for mutants of E. coli (116) that respond to either vitamin B_{12} or methionine. The pseudovitamins approach (117) or equal vitamin B_{12} in activity (116). It was suggested earlier (118) that the B_{12} sparing effect on the PAB requirement of an auxotroph of E. coli is the result of participation of PAB in B_{12} synthesis as a catalyst or a structural precursor of the benzene ring; however, the fact that the pseudovitamins contain the purine ring instead rendered the structural precursor theory unlikely. In Euglena gracilis (119) the pseudovitamins show the same order of activity as does vitamin B_{12}, while the amorphous series (114), as well as ammonia cobalichrome (ammonia substituted vitamin B_{12}) (120) are only half as active as vitamin B_{12}. Ammonia cobalichrome has been shown to possess 2.5 times the activity of B_{12} for L. lactis, 1.5 times for E. coli, and equal activity for chicks. Another B_{12}-like substance called B_{12f} (for feces) (121, 122) is inactive for the rat or chick but is active for Euglena, E. coli and L. leichmannii. Other reports on this substance conflict; Robbins et al. (119) find this form to be about twice as active for Euglena as for L. leichmannii, whereas Lewis et al. (121) report it to be equally active for the two organisms. Vitamin B_{12f} appears to have a more intense absorption maximum at 278 mμ than does pseudovitamin B_{12} (122). The authors suggest, however, that the differences may be due to impurities in the B_{12f} preparations and believe that the two forms may be identical on the basis of similar inactivity for the rat and chick, as well as of chromatographic behavior.

Still another vitamin B_{12}-like substance is found in autolysates of chick, turkey, and turtle blood, as well as in liver concentrate. These materials show three zones ($R_F = 0.6, 0.8, 1.0$), in addition to vitamin B_{12}, active for L. leichmannii 4797 in bioautographic analysis using water-saturated butanol as the developing solvent (123). The slower and the faster zones are

presumably identical to hypoxanthine desoxyriboside and thymidine, respectively. The substance with R_F 0.8 is unidentified; whether or not it is identical with any of the pseudovitamins is not known. These factors would evidently increase the apparent vitamin B_{12} values when the samples are assayed with *L. leichmannii*; however, blood from the cow, sheep, and pig contain only one factor, namely, vitamin B_{12}. Because of the complexity and multiplicity of the vitamin B_{12} group, it may be necessary to await further determination of the respective structures before definite assurance of identities may be enjoyed.

The suggestion was made (121) that vitamin B_{12f}, which appears to be elaborated by intestinal bacteria, may be the factor required by cobalt-deficient sheep. Microbiological assay of the rumen contents of sheep gave values three times those obtained by rat growth. If this suggestion is correct, the discovery of these new forms may represent an important advance in the knowledge concerning vitamin B_{12} and the anemias.

Thymidine can replace B_{12} in the growth of certain strains of *L. leichmannii* only in the presence of guanine. Omission of guanine causes adenine to inhibit growth markedly in the presence of vitamin B_{12} and slightly in the presence of thymidine. Maximal growth can be achieved by supplementing vitamin B_{12} with adenine, guanine, and uracil (124).

Heating gastric juice with vitamin B_{12} leaves a part of the vitamin in a form unavailable to microorganisms. The binding factor is reported (125) not to be identical with the intrinsic factor of Castle (*cf.* 126). Sow's milk also contains a factor which can bind B_{12}, rendering it unavailable to certain assay microorganisms (127). A binding factor(s) in serum has been fractionated by paper electrophoresis (128). Another source of the factor(s) is pig stomach (129). Preparations from this source inhibited the utilization of the vitamin by *E. coli*, whereas another protein, lysozyme, had no effect.

A further study has been made (130) of the effect of diffusion of oxygen into the medium upon the precision of the assay of vitamin B_{12} by *L. leichmannii*. Earlier findings are confirmed (see, for example, *U. S. Pharmacopeia*, XIV). Sodium nitrite produces a progressive increase in response of *L. leichmannii* to B_{12} at 2 mg. to 2.0 g. nitrite per 0.2 μg. of B_{12} (131). However, the sodium bisulfite sparing effect on B_{12} is independent of its concentration within the concentration ranges usually employed. The dosage-response curves of *L. leichmannii* for the determination of vitamin B_{12} are logarithmic. Addition of up to 15 mg. NaCl per ml. of basal assay medium changes the curve to an arithmetic type (132). Methods have been proposed (117, 133) for vitamin B_{12} assay by means of a mutant of *E. coli* (134). Interference by methionine is not serious until its presence exceeds that of B_{12} by 50,000 fold (117).

OTHER KNOWN FACTORS

α-Ketoglutarate, pyruvate, and lactate can spare the requirement of PAB for the growth of *L. arabinosus*. Malonate and fumarate exert a smaller sparing action, whereas citrate is inhibitory (135).

The function of PAB in the synthesis of purines via conversion of 4-amino-5-imidazole carboxamide has been confirmed in a study of a PAB-requiring mutant of *E. coli* (136). Methionine and vitamin B_{12}, both of which are capable of sparing the PAB requirement of the mutant, can depress the accumulation of the imidazole derivative, but are not completely equivalent in this action.

Growth of *Neurospora sitophila*, which has been used frequently for vitamin B_6 assay, is enhanced by high concentrations of B_1. This is thought to be due to the fact that B_1 inhibits the destruction of B_6 and that B_6 in turn inhibits B_1 biosynthesis (137).

Pyridoxal phosphate has been demonstrated as a coenzyme of D-serine dehydrase. However, no evidence for the corresponding activity of this coenzyme in L-serine dehydration (deamination) could be obtained (138).

In a basal medium of casein hydrolysate, salts, citrate, and succinate as the carbon source with high levels of NaCl and $MgSO_4$, the growth of a *Vibrio* species is stimulated somewhat by ribonucleic acid or its pyrimidines, uracil and cytosine (139). A *Micrococcus* species requires thiamine and is stimulated by purines and pyrimidines. A *Sarcina* species requires thiamine, pantothenic acid, nicotinic acid, and biotin for growth and is stimulated by folic acid and PAB, uracil, choline, and pyridoxal. Yeast extract was the most effective material tested.

Orotic acid has been shown as a growth factor for *Lactobacillus bulgaricus* 09 (140, 141). Among several intermediates in the chemical synthesis of orotic acid tested, only ureidosuccinic acid possesses any activity (about 10 to 20 per cent of orotic acid). Although urea shows similar behavior, evidence was found that the entire molecule of ureidosuccinic acid is utilized by the organism in pyrimidine synthesis (142). From growth and tracer studies uracil was found to be incorporated after greatly prolonged incubation (143). Based on these findings as well as on the inactivity of other nucleotides as growth factors for *L. bulgaricus*, the authors suggested that nucleic acid synthesis does not usually involve the pyrimidine nucleosides and nucleotides as intermediates, but instead involves the participation of orotic acid nucleosides and an orotic acid nucleotide (142). However, it is believed also (143) that spontaneously occurring mutants of the organism may often arise, in which the biosynthesis of nucleotides may follow various other pathways.

An improved medium has been developed for the assay of pteroylglutamic acid with *L. casei*. This medium can be used also for the determination of riboflavin, nicotinic acid, pantothenic acid, biotin, and tryptophan. The bound form of nicotinic acid present in cereals is found to be less active than the free form (144).

CARBON SOURCES

In a study of the nutrition of *Mycobacterium tuberculosis* (145, 146, 147) it has been found that only glycerol, glucose, and possibly maltose can sup-

port its growth, but not fructose, galactose, mannose, or 20 other carbo-
hydrates tested. Various acids tested (pyruvic, acetic, oxalacetic, oxalo-
succinic, α-ketoglutaric, and lactic) can replace glycerol or glucose as the
carbon source, although a number of other Krebs cycle intermediates,
glutamic and aspartic acids, and alanine are not effective as carbon sources.
These results suggest that perhaps some cyclic process may be involved in
the carbohydrate metabolism of this organism. However, Embden-Meyerhof
glycolytic intermediates do not support growth appreciably. The fact that
pyruvic and lactic acids stimulate the rate of growth to a greater degree
than does glucose suggests that the conventional glycolytic scheme perhaps
plays a less important role, and points to the presence of a more efficient
terminal oxidation pathway in this species.

Resting cells of *A. suboxydans* have been observed to oxidize many com-
pounds, such as dihydroxyacetone, pyruvic acid, formaldehyde, lactic acid,
and others (148), but these compounds cannot be used as the carbon source
for growth (149, 150). Members of the citric acid cycle cannot be utilized
either for growth or as oxidizable substrates (148, 150), and it is believed that
the cycle plays an insignificant role in this organism.

When acid production is used as the growth criterion, Rose *et al.* (151)
found that *L. bifidus* utilizes the disaccharides lactose and maltose, but not
sucrose, more readily than the monosaccharides, glucose, and galactose.
The straight rod variants of *L. bifidus* show the opposite behavior. Several
strains of *L. acidophilus* utilize glucose equally or slightly more readily than
lactose. During the growth of *L. bifidus* on lactose, glucose and galactose
accumulate in the medium. The straight rod mutant of *L. bifidus*, on the
other hand, utilizes lactose completely. The preferential use of the disac-
charides in certain strains may be the result of the extra energy available
during the cleavage of the saccharide linkage in the presence of a suitable
enzyme. Nonutilization of disaccharides, on the other hand, may result
from low concentration or absence of the enzyme.

When xylose replaced glucose in a synthetic medium for *L. pentosus*,
a requirement for a yeast supplement was created (152). The existence of a
"xylose factor" in yeast extract was thus proposed. It was later observed
(153) that the "xylose factor" effect could be duplicated by the addition of
(in the order of decreasing activity) glucose, mannose, water soluble liver
fraction, maltose, lactose, or fructose in a synthetic medium containing
xylose or arabinose as a carbohydrate source for the growth of *L. pentosus*
124–2 (*L. plantarum*). Sucrose was inhibitory to glucose. The activity in
liver extract could not be separated by ion exchange, charcoal adsorption,
or paper chromatography from the sugar present. Glucosazone was isolated
from an active fraction of liver extract. It was also found (154) that when
S. faecalis is grown in media in which neutralized gluconolactone is sub-
stituted for glucose, a small amount of glucose or yeast autolysate becomes
necessary. The conclusion, that the sugar content in natural materials is
responsible for the activity in both organisms, requires further study.

Two new mutants of *N. crassa*, C-24 and T-2307, utilized formate or formaldehyde for growth (155). Adenine supports limited growth of both strains. A mixture of adenine plus methionine for C-24 or L-serine for T-3207 supports the growth of the organisms in place of formate or formaldehyde. Other compounds related to C_1 metabolism (choline, glycine) in animals studies show stimulating activity but cannot replace formaldehyde or formate completely.

The growth of a strain of *Neisseria meningitidis* requires CO_2 supplementation (156). The latter can be replaced by yeast extract, but not by any known vitamins, pyrimidines, or purines tested. The unknown factor(s) is soluble in alcohol, acetone, or water. It is stable to autoclaving at pH 1.8 or 11.7 but labile to ultraviolet light. However, it is not identical with lipoic acid. The fact that lipoic acid cannot substitute for CO_2 suggests that this catalyst does not operate in the C_1-fixation system in this organism.

The CO_2 requirement in a *Penicillium chrysogenum* strain could not be replaced by yeast extract (157), mold extract, or other complex materials but could be eliminated by corn steep medium or oxalacetic acid. The latter may be decarboxylated to yield CO_2. The amount of $C^{14}O_2$ fixed by the mold in a synthetic medium varied with the salts, nitrogen source, and the other composition of the medium. Most of the CO_2 fixed was converted to aspartic and glutamic acids and arginine.

The simultaneous addition of asparagine, arginine, uracil, adenine, and guanine can reduce CO_2 fixation in growing *Serratia marcescens* to 7 per cent of the maximum that is possible in a glycerol-ammonium salt medium. Each replaces a definite proportion of total CO_2 fixation and is effective alone. The amount by which CO_2 fixation is reduced is approximately equivalent to the amounts of these substances synthesized by the bacteria (158).

D-Lactic acid is reported to be a growth stimulant for *L. casei* (159).

AMINO ACIDS AND AMIDES

In a continuation of studies on D-amino acid utilization in lactic acid bacteria, it appeared (160) that D-(as well as L-) aspartic acid is an essential metabolite for *Lactobacillus brevis*, *L. lycopersici*, and *Leuconostoc mesenteroides*. This conclusion was based on the ready utilization of the D-isomer for growth and its occurrence in hydrolysates of the bacterial cells, in concentrations up to 3 per cent of the total protein. *L. arabinosus* does not require D-aspartic acid, nor is the compound found in the cells.

D-Glutamate is a more effective replacing agent for L-glutamate than is α-ketoglutarate. This apparently is because of the presence of a racemase system rather than to conversion through deamination and transamination (161). The racemase activity can be demonstrated both in acetone powders of the organism and in dried cells (162). On the other hand, higher levels of racemic forms of amino acids show inhibition in the growth of *Brucella abortus* (163). The effect has been traced to the presence of the D-forms. Various amino acids exhibit different degrees of inhibition, with D-phenylalanine

and D-methionine producing the greatest inhibition. The more interesting fact is that, when a CO_2-requiring strain is grown in suboptimal amounts of added CO_2, the inhibitory effect of the racemic forms is more pronounced. No reversal experiments have been attempted.

The appearance of nonsmooth variants of *Brucella* from smooth strains of the organism was shown earlier (164) to be stimulated by the presence of D-alanine in the medium; L-alanine had no such effect. More recently (165), the production of alanine in the medium has been correlated with the use of D-asparagine for growth, and it has been found that L-asparagine formed no alanine and favored the maintenance of smooth colonies during incubation periods of a week or more. Later, mucoid mutants appeared even in the L-asparagine medium, and another amino acid accumulated which was believed to be valine. It is evident that the configuration of substrate amino acids may have an important influence upon population characteristics in these bacteria.

It has been suggested that anthranilic acid can serve as a precursor, via indole, of tryptophan in certain organisms, for example, *L. arabinosus* and *Neurospora*. However (166), although indole and anthranilic acid were active as reported (167, 168), none of the 19 possible intermediates in the pathway of indole synthesis from anthranilic acid tested supported the growth of *L. arabinosus* 17-5 in an acid-hydrolyzed casein medium. The question of cell-permeability was not ruled out in these experiments, but attempts to show indole or tryptophan formation during anthranilic acid utilization by resting cells were unsuccessful. Similar experiments with indole also failed to produce tryptophan. In growth experiments, cells produced from anthranilic acid or indole in place of tryptophan contained significantly less tryptophan, and it was concluded (166) that the route of synthesis of this amino acid in *L. arabinosus* is different from that in the mold *Neurospora*.

In a study of the nutrition of threonineless mutants of *E. coli* (obtained by ultraviolet irradiation), three separate growth responses were observed [Umbarger (169)]. One group responded only to L-threonine. The second group responded to D-threonine, (or D-allothreonine), α-ketobutyric, α-aminobutyric or α-keto-β-hydroxybutyric acids, L-isoleucine or its keto and α, β-dihydroxy analogues. The third group responded to all the compounds supporting groups one or two, and, in addition, were able to utilize DL-homoserine. However, only DL-homoserine and L-threonine could support maximal growth of the last group. An unknown compound accumulated in filtrates of cultures of the first group during growth on limiting amounts of L-threonine. This compound supported the growth of the third group. Amos & Cohen (170) observed similar results, and noted that the strain which requires D-threonine is inhibited by L-serine. These findings are in line with the results reported by Teas & Garner (171, 172) that threonine and methionine have a common precursor, perhaps homoserine.

When *E. coli* strain B (173) grows in a medium containing radioactive

sulfate as the sole sulfur source, radioactive methionine and cysteine-cystine have been found in the harvested cells. Cells grown in radioactive sulfate and nonradioactive homocystine or methionine contain no labeled methionine, while most of the S^{35} is in cysteine-cystine. This result confirms the hypothesis that cystine metabolism follows two pathways in these cells.

In a methionine-requiring mutant (1–344) or *E. coli* (174), inorganic sulfate can not be used for methionine synthesis, and methionine sulfur is not converted to other sulfur compounds at any concentration of methionine in the medium. Maximum growth requires nonmethionine sulfur along with methionine. *Pasteurella pestis* strain A1122 (175) requires phenylalanine, isoleucine, methionine, and thiosulfates as nitrogen and sulfur sources for growth. In the presence of methionine, thiosulfate is replaceable only by either cysteine, sulfide, or sulfite, and in the presence of thiosulfate, methionine, is replaceable only by homocysteine or cystathionine. This fact indicates that this organism is not able to synthesize cysteine and methionine from inorganic sulfate or to interchange the sulfur atom in methionine and cysteine, and it evidently lacks the appropriate enzymes that are present in strain B of *E. coli* (173). A "spontaneous" mutant was reported which utilized sulfite, thiosulfate, sulfide, or cysteine as the sole sulfur source (175). It was suggested that a gain mutation may have occurred at the cysteine→cystathionine site. This change would enable the mutant to synthesize methionine from any of the above four compounds; however, it was still unable to use methionine, homocysteine, or cystathionine as the sole sulfur source. The latter fact perhaps points to the irreversibility of cysteine→methionine synthesis in this organism. *Brucella suis* can satisfy its sulfur requirement on either L-cystine, L-cystathionine, or L- or D-methionine in an asparagine-lactate medium (176). DL-homocystine or its reduced form and DL-homocystine thiolactone also can be utilized to a degree related to their methionine content. However, purified preparations of DL-homocystine were not utilized except in the presence of small quantities of supplementary methionine.

Several studies of amino acid biosynthesis in yeast have been carried out using C^{14}-glucose (177, 178, 179), pyruvate (180, 181), and acetate (177 to 186). From these studies it is evident that any one of the foregoing substrates is readily convertible into protein, and the patterns of radioactivity in the isolated amino acids furnish considerable information regarding possible routes of biosynthesis of amino acids. With acetate as substrate, the ornithine chain of arginine was regarded (185) as arising directly from α-ketoglutarate, which in turn appeared from its C^{14} content to be formed via the Krebs cycle. Lysine was pictured as being derived from α-amino-adipic acid [(186); cf. also (187) for the origin of this amino acid in *Neurospora*]. Phenylalanine and tyrosine were first considered (177, 178) to arise from acetate via glucose, followed by cyclization to produce the aromatic ring; however, this opinion has been changed (179) and it is not clear from present results what the exact pathway may be to the aromatic acids. In

E. coli, shikimic acid has been indicated as a precursor of the aromatic ring (188, 189), and it is suggested (179) that a heptose such as sedoheptulose (190) may serve as a precursor of shikimic acid or the aromatic ring in yeast. Other data (191) indicate that, when pyruvate is the carbon source, C_4 acids may function as intermediates in tyrosine formation. Aspartic acid may arise from acetate via Krebs cycle type reactions (183) or from a C_2-C_2 condensation (181); from pyruvate, a C_3-C_1 condensation prevails, accounting for up to three-fourths of the total aspartic acid formed (181). Glutamic acid is produced in enormous amounts from pyruvate during short growth periods (180); up to one-half of the total growth of the organism may be represented by this amino acid alone. The distribution of C^{14} in glutamic acid suggests the participation of Krebs cycle-type condensations, starting both from pyruvate (192) and acetate (183). From acetate, it would appear that the carboxyl group is incorporated into the carboxyl groups of many of the amino acids (183). Histidine carboxyl is an exception, arising from the methyl group of acetate. Other data (193) sugggest that oxalacetate or another C_4 acid may serve as an intermediate in the formation of the histidine side chain. Thus, although there is some controversy (194, 195) over the quantitative importance of the Krebs cycle in yeast, especially with respect to energy metabolism, there is good evidence that compounds common to the cycle may function as precursors of several of the amino acids.

Strong stimulation of *L. casei* growth has been provided by serine in the presence of Ca^{++} (159). No similar effect could be demonstrated among nine other amino acids tested, and no explanation could be provided for the unique relationship between serine and calcium. A common antagonism between sodium and calcium was noted also.

An enzymatic casein hydrolysate supplement, required (196) for the optimum growth of a nonpathogenic Reiter strain of *Treponema*, can be replaced by amino acids with a number of other known growth factors and crystalline albumin (197). It becomes evident that the nutritional requirements of this organism do not differ qualitatively from those of other fastidious organisms.

In a medium composed of Difco casamino acids (acid-hydrolyzed casein) as nitrogen source, glucose as carbon source, and salts, the rate of growth of *Mycobacterium phlei* is retarded. Addition of beef heart infusion increases the rate. In the presence of bovine serum albumin, the Tweens, the phospholipid fraction of egg yolk, and oleic acid also enhance the growth (198). However, the casein hydrolysate can be replaced by a mixture of L-glutamic acid, NH_4Cl, L-arginine, L-proline, and histidine for rapid growth (199).

Studies have been continued on the induction of maltase in *S. cerevisiae* (200, 201). The effect of 23 amino acid analogues on enzyme formation was compared with their influence on growth and the behavior of the free amino acid pool. Ability to suppress growth was found coupled with capacity to inhibit enzyme formation in nongrowing cells. Complete and specific reversal

was achieved by adding the corresponding homologous amino acid. The presence of one analogue could prevent the incorporation of all identified components in the free amino acid pool. However, no evidence for a direct, amino acid-independent transformation of complex precursor into active enzyme was obtained.

It was pointed out in last year's review (2) that the response of organisms to peptides may result from two influences: direct utilization, or preliminary hydrolysis followed by utilization of the constituent amino acids. The first of these might be reflected in enhanced growth of the organism caused by preferential use of the intact peptide. Apart from this, it has been shown that in *L. casei* (202), when D-alanine was present in the medium it inhibited the utilization of the L-isomer; however, D-alanine had no effect on L-alanine peptides. A similar effect was then suggested for other systems, i.e., an antagonism among certain related amino acids that may not be experienced when peptides are employed instead. The destructive action of tyrosine decarboxylase (203) upon free tyrosine, but not on its peptides, was also noted and offered as an explanation of the greater response of *S. faecalis* to tyrosine peptides.

ANTIMETABOLITES

An interesting variation in the approach to antimetabolite action and chemotherapy has been provided by the postulate (204) that 1,2-dimethyl-4,5-diaminobenzene (DMDA, structure II) is a metabolic precursor for both riboflavin and vitamin B_{12}. A series of 35 analogues of this structural unit has been prepared, which has aimed in part at the preparation of "aggregate molecules" that might inhibit the biosynthesis of both metabolites (205). The halogen analogues (structure III and IV) usually showed inhibitory action in *Staphylococcus aureus*. Structure IV was competitively antagonized by DMDA.[2] Acylation or alkylation of one amino group of any one of structures II, III, or IV gave an inert compound. However, the replacement of one amino group by a hydroxyl or of both by nitro or hydroxyl yield active growth inhibitors.

The theoretical approach was considered justified by the facts that: (a) the dichlorodiaminobenzene inhibited best those organisms that synthesized both riboflavin and vitamin B_{12}, whereas other organisms that required either metabolite for growth were more resistant, and those that required both were not affected at all; (b) two "aggregate" molecules, made from 1,2-dichloro-4,5-diaminobenzene and sulfonamides (inhibitory analogues of PAB), were much more active inhibitors than the parents; (c) higher animals, as expected, were rather resistant to these analogues, since they require both vitamins preformed in the diet; for example, 1,2-dichloro-4-(*p*-nitrobenezenesulfonamido)-benzene caused half maximum inhibition in *S. aureus* (206) at 3 μg./ml., in the presence or absence of DMDA and PAB. However, the LD_{50} in mice was 120 mg./kg.

In extension of this reasoning Woolley has made the intriguing sugges-

II

III
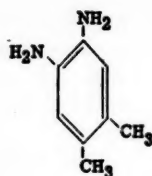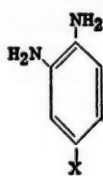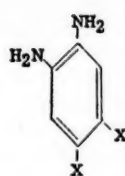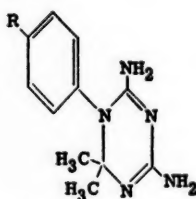
IV

(a) R = COOC$_2$H$_5$

(b) R = Cl

(c) R = H

V
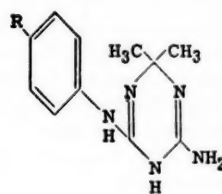
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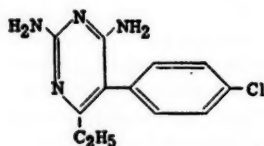
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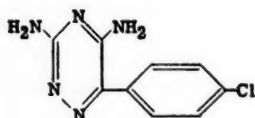
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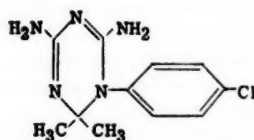
IX

tion (207) that tissues, e.g., spontaneous tumors, which may synthesize significant amounts of vitamin B$_{12}$, may be suitable "substrate" cells for the selective application of the foregoing types of inhibitors. No experimental work was presented to test the idea, but the advantages of such an approach even if only partly successful, are obvious, and this provocative outline merits close attention and study.

In a search for new antimalarials, a series of diamino triazine derivatives has been synthesized and tested for antimetabolite activity (208). Many compounds of structure V inhibit *S. faecalis* and *L. arabinosus*, whereas

those of structure VI are relatively inert. High concentration of the hydro-chloride of Compound Vb also inhibits *L. casei* and *L. citrovorum* against the folacin-CF group. The inhibition induced by compounds of structure V is different from that induced by 4-aminopteroylglutamic acid. The new inhibition can not be reversed by pteroylglutamic acid, adenine, or guanine but is reversed by CF, leucovorin, dihydropteroylglutamic N^{10}-formyl pteroylglutamic acid, certain components of nucleic acid, or high concentra-tion of ascorbic acid. Several compounds of structure V also show activity against experimental avian malaria.

Other pyrimidine and triazine derivatives also have been tested (209, 210). Compound VIII is a competitive antagonist of folic and folinic acids in the growth of *L. casei* and of folinic acid (leucovorin) in the growth of *L. citrovorum*. The inhibition index in the latter system is about 20,000 at half maximal growth. With VIII, as with VII and IX, the minimum con-centration for inhibition of *S. faecalis* is several hundred-fold greater when folinic acid is supplied than when the organisms are grown with folic acid. In *L. casei* folic and folinic acids are essentially equivalent over a wide range of concentration of the inhibitors. Also in *L. casei*, 5-benzyl-, 5-phenoxy-, and 5-phenyl-2,4-diaminopyrimidines are powerful antagonists of folic acid (211). In *L. citrovorum* they antagonized CF (leucovorin was used). The inhibition ratio of these pyrimidines to folic acid in *S. faecalis* was about 16, whereas that in CF was much higher. The diaminopyrimidines employed in these studies were viewed (211) as distant relatives of folic acid, possessing in common the 2,4-disubstituted pyrimidine structure, as well as benzene rings and a generally similar geometric configuration. It was sug-gested that analogues of closer resemblance to the metabolite might be less capable of species differentiation, since they would possess more points of contact that are common to the protein receptor groups of all species. Since these receptor groups determine principally the dissociation of the anti-metabolite-enzyme complex, it was hoped that the estrangement of several portions of the analogue molecule would give rise to complexes that would vary in degree of dissociation from species to species, and thus gain value, through selective action, as chemotherapeutic agents.

On the other hand, in the nutrition of *Tetrahymena geleii*, a folic acid antagonist, aminopterin (4-amino PGA) can replace PGA[2] for growth (212, 213, 214). Methopterin (10-methyl PGA) is less effective and ame-thopterin (4-amino-10-methyl PGA) is inert. A strain of *S. faecalis* resistant to amethopterin can utilize certain PGA antagonists (except for 9-methyl derivatives) for growth in lieu of PGA (215, 216). Aminopterin is even as effective as PGA in reversing the toxicity of A-ninopterin (4-amino-9-methyl PGA) and A-denopterin (4-amino-9, 10-dimethyl PGA) in the growth of this strain. The requirement of PGA (or pteroyltriglutamic acid or pteroic acid) but not of CF in this strain is lower than that in the parent strain (215).

These experiments demonstrate that this organism, in contrast to other bacteria, is able either to convert some antagonists into folic acid or else to

utilize the analogues *per se* in the formation of coenzyme analogues. Assays of the folic acid contents of these organisms should establish which of these possibilities is correct.

Isonicotinic acid hydrazide inhibits the formation of indole in *E. coli*. The activity can be recovered by treating the cell suspension with pyridoxine (217). It is suggested that the drug may act on tryptophanase as an antimetabolite against pyridoxal phosphate, which has been reported as the coenzyme of tryptophanase by Wood *et al.* (218). It was also found that this drug inhibits amino acid decarboxylation in *E. coli* (217); the latter system is likewise pyridoxal phosphate-dependent. Isonicotinyl hydrazide may be assayed microbiologically (through its inhibitory behavior) in *Mycobacterium butyricum* with ± 15 per cent accuracy at a level of 5 µg. or more of the compound per ml. It is claimed that the method is more nearly specific than chemical methods (219).

5-Fluoro-, 5-bromo-, and 5-chloronicotinic acids inhibit the growth of *Staphylococcus aureus*, *L. arabinosus*, *Proteus vulgaris*, and *E. coli* (220), while 2- and 6-fluoronicotinic acids are not inhibitory. Nicotinamide, its riboside, ribotide, and diphosphopyridinenucleotide (DPN) are less efficient in reversing the inhibition than the acid itself. The glycolysis by washed suspensions of nicotinic acid deficient cells of *L. arabinosus* and *S. aureus* and the terminal respiration of *P. vulgaris* are not inhibited by the fluoro-analogue. However, the stimulation of these processes by added nicotinic acid (221) is inhibited. These results suggest that the growth inhibition is due to a block of the utilization of nicotinic acid for DPN^2 synthesis. It has been verified in resting cell suspensions of *L. arabinosus* and *S. aureus* that DPN synthesis is actually strongly inhibited by the analogue (220). Among a series of 37 derivatives of pyrazines and pyridines tested, pyrazinamide, pyrazinoic acid, and 2-sulfanilamide-5-nitropyridine were found to be nicotinamide antagonists for *L. arabinosus* (222). The inhibition indices were about 100,000 to 250,000. No reversal test with PAB versus 2-sulfanilamide-5-nitropyridine was carried out. Pyrazinamide did not significantly affect the growth of rats and chicks.

4-(Imidazolidone-2)-caproic acid, homobiotin, norbiotin, and hexahydro-2-oxo-4-hydroxybutyl-1-furo-(3,4)-imidazole inhibited growth of virulent tubercle bacilli in low concentrations. Biotin was able to reverse the inhibition (223), with inhibition indices (for complete reversal by biotin) ranging from 120 to 260. Desthiobiotin and oxybiotin could replace biotin as reversing agents to varying degrees. However, aspartic acid and sorbitan monooleate had no effect on the bacteriostatic activity of these analogues. When tested in chick embryos, 4-(imidazolidone-2) caproic acid was ineffective in checking the course of infection in embryos infected either intravenously or via the chorioallantoic membrane.

The respiration and growth of *Bacillus subtilis* are inhibited by *cis*-vaccenic, stearic, and linoleic acids. The inhibition is transient at certain levels of the acids. Respiration and growth ultimately proceeded at rates

equal to or even greater than those of untreated controls. Bacterial cells which have recovered from the initial inhibition by any one acid were found to be resistant to further addition of the acid or any of the other acids. It was suggested that the resistance of acid treated cells was caused by the development of an adaptive enzyme (224).

An antagonism by choline toward inositol was noted in *Saccharomyces carlsbergensis*, at a choline to inositol molar ratio of 0.3 (225). Maximum inhibition occurred at a ratio of 15. Similar inhibition was observed with dimethylaminoethanol, but not with ethanolamine, serine, betaine, methionine, or choline phospholipides. The inactivity of the phospholipides is likely because of the barrier of permeability. The failure of betaine and ethanolamine to inhibit growth suggests that their metabolic pathways may be different from those of choline.

The utilization of glutamic acid in *L. arabinosus* 17–5 was competitively inhibited by α-methylglutamic acid and one of the diastereoisomeric racemates of β-hydroxyglutamic acid, among eight other related compounds tested (226). None of the compounds was a glutamine inhibitor.

Lysine polypeptides can agglutinate cells of a few species of bacteria at 5–50 μg./ml. (227). Growth is also inhibited by as little as 4 μg./ml. in *E. coli*. Addition of 1 to 10 μg./ of ribonucleate or desoxyribonucleate or L-glutamic polypeptide reverses the inhibition.

The activity of a synthetic sample of polylysine peptide containing an average of 85 lysine residues per molecule was compared (228) with a natural antibacterial basic protein from calf thymus (229) in a system comprising bacteriophage and its host, *Clostridium madisonii*. Both the antibacterial and antiviral properties were about four times higher in the synthetic sample than the natural product. From the fact that the tissue peptide contained 30 per cent lysine, it was possible to account for the activity as being due to the lysine moiety of the molecule. It has been shown further that both peptides combine stoichiometrically with desoxyribonucleic acid at pH 6.5. The peptides combine with ribonucleic acid in varying proportions depending upon environmental conditions (228). It seems that the antimicrobial property of the peptides may be due to the disturbance of nucleotide metabolism of the organism.

It was reported that propionate inhibition in *S. faecalis* 8043 can be reversed by acetate (230). The amount of propionate required to inhibit the microorganism was higher in the presence of pantothenate than in its absence, which suggested that the organism was capable of synthesizing the vitamin. This synthesis was verified by direct assay, but it is a surprising observation. Within the reviewers' experience (231), *S. faecalis* 8043 constitutes no exception to the general experience from many laboratories, that lactic acid bacteria do not synthesize pantothenate. In the absence of further definite evidence regarding the identity and homogeneity of the culture used, the present authors view with some reservation the otherwise plausible interpretation of propionate action, i.e., that it combines with

CoA (synthesized by the organism) to form propionyl CoA, and thus interferes with the oxidative decarboxylation of pyruvate to form acetate. Sodium propionate is also reported (231a) to be a bacteriostatic and fungistatic agent against 21 common microorganisms.

Nitrous oxide competitively inhibits nitrogen fixation in *Azotobacter vinelandii*. The dissociation constant for the enzyme inhibitor complex is about 0.08 (232, 233). The authors believe that N_2O inhibits some reaction prior to the formation of ammonia, since the latter prevents N_2O inhibition. Finally, it is suggested that this inhibition offers indirect support for the theory that nitrogen is oxidized prior to its reduction to ammonia in the organism.

The intestinal contents and mucosa contain a substance(s) which inhibits *S. agalactiae* to a degree equivalent to a solution of 70 µg. of penicillin per ml. (234).

Lineweaver & Burk (235), in their derivation of the Michaelis-Menton equation, divided the kinetics of enzymic reactions into 7 categories. The variables were similarly utilized and simplified by Shive (236) in the development of "inhibition analysis." This method of attack has been open to some criticism (237, 238).

In this theory, the fundamental assumption is

$$E + S \leftrightarrow ES \rightarrow P + E$$
$$E + I \leftrightarrow EI$$
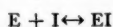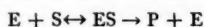
where

E = enzyme	P = product
S = substrate	ES = enzyme-substrate complex
I = inhibitor	EI = enzyme-inhibitor complex

It is correct in many systems. However, when working with growth systems there is a question of whether or not the possibilities other than case 1 in Lineweaver & Burk's treatment should be considered, among them, case 7, namely,

$$S \rightarrow S' \text{ (steady state)}$$

Then

$$S' + E \leftrightarrow ES' \text{ (active)}$$
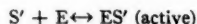
requires special attention. As in the original statement (233), the concentration of active intermediate is not directly proportional to (E)(S) but to (E)(S'). The reaction $S \xrightarrow{K_1} S'$ may either be physical or chemical. In inhibition, a similar reaction $I \xrightarrow{K_2} I'$ and then $I' + E \leftrightarrow EI'$ may occur. K_1 and K_2 may become limiting. Thus the over-all result might not reveal the nature of the inhibition. In other systems, the interrelationships among I, S, ES, and EI may be further complicated by possible conversion of I to S or I to a biologically active coenzyme (52, 212, 213), expressible as follows:

S
↗
I
 ↘
| I′ (active inhibitor)
↓
Ia (active catalyst)
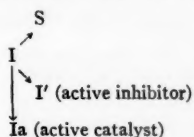
Then the actual inhibition or stimulation depends upon the rate of the above reactions. Moore & Boylen (239) analyzed the growth inhibition and found three different types of response to an inhibitor. Only one of the three types can be interpreted as representing a simple inhibition of the rate of a single reaction sequence. Their observation deserves further study. The general idea of "inhibition analysis" is helpful in understanding the complex relationships that exist in tissues. However, their very complexity necessitates special care in the interpretation of results obtained through the use of oversimplified equations.

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METABOLISM OF MICROORGANISMS[1,2]

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As a result of space limitation, a complete coverage of the literature in the field of microbial metabolism has not been attempted in the following review. This review has been limited to a consideration of publications during the period January 1952 to February 1953 which dealt with fatty acid, pyruvate, amino acid, and steroid metabolism. In some instances earlier material which had not been covered in previous reviews or was necessary for a logical presentation of the subject has been included. Recent developments in the fields of purine and pyrimidine metabolism have been summarized in several review articles (1, 2, 3) and, therefore, were not included in this discussion.

FATTY ACIDS

Acetate oxidation.—The quantitative importance of the conventional TCA[2] cycle in the oxidation of acetate by microorganisms has been challenged by several investigators as a result of their failure to observe the expected incorporation of isotope into various TCA cycle intermediates when these were supplied as unlabeled carriers during the oxidation of labeled acetate. Thus, in experiments with cell suspensions of *Aerobacter aerogenes*, Ajl & Wong (4) observed that following the oxidation of 2-C^{14} acetate in the presence of unlabeled carrier TCA compounds, the specific activity of the carboxyl carbons of any of the reisolated carriers is much lower than that of the respired CO_2. Similar results were obtained by Krebs *et al.* (5) in experiments with bakers' yeast (which had been pretreated with dry ice to overcome permeability difficulties) and in experiments with cell-suspensions of *Micrococcus lysodeikticus* (6).

It should be emphasized that results of isotope experiments obtained by

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[1] The survey of the literature pertaining to this review was concluded in February, 1953.

[2] The following abbreviations are used: ADP for adenosinediphosphate; AMP for adenosinemonophosphate; ATP for adenosinetriphosphate; CoA for coenzyme A; acetyl-P for acetyl phosphate; CoA-PP for coenzyme A-pyrophosphate; DAP for $\bar{a}$,ϵ-diamino-pimelic acid; DNP for dinitrophenol; DOPA for dihydroxyphenylalanine; DPN for diphosphopyridine nucleotide; DPNH for diphosphopyridine nucleotide (reduced form); Pi for inorganic phosphate; PAB for para-amino benzoic acid; POB for para-hydroxy benzoic acid; POF for pyruvate oxidase factor; PP for pyrophosphate; PTA for phosphotransacetylase; TCA for tricarboxylic acid; TPN for triphosphopyridine nucleotide; TPNH for triphosphopyridine nucleotide (reduced form).

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the carrier technique can be used to establish or exclude a given metabolic pathway only insofar as each of the added carrier compounds is in complete equilibrium with the corresponding enzymatically produced intermediates. In this connection the studies of Saz & Krampitz[4] (7) are of special significance. Using lysed preparations of *M. lysodeikticus* they have confirmed the previous findings (6) that during the oxidation of 2-C^{14} acetate in the presence of added TCA intermediates, the specific activity of the respiratory CO_2 is many-fold higher than that of the carboxyl carbons of any of the re-isolated carriers. On the other hand, in experiments performed in the absence of carrier compounds, in which sufficient enzyme was used to permit isolation and specific activity determinations of the endogenous TCA intermediates, it was found that labeled acetate, when added in amounts sufficient to give a half maximal rate of oxidation, was in complete isotopic equilibrium with all members of the TCA cycle, and with the respired CO_2. These results offer definitive evidence that added carrier TCA compounds are not always in rapid equilibirium with the corresponding metabolically produced intermediates.

Comparable results were obtained in studies with soluble, partially purified enzyme preparations from *Lactobacillus arabinosus* (8). These preparations catalyze the over-all reaction:

$$\text{L-malate} \rightleftarrows \text{lactate} + CO_2 \qquad\qquad 1.$$

When the reaction is carried out with L-malate labeled in both carboxyl carbons with C^{14} and in the presence of a pool of unlabeled pyruvate, relatively little isotope is incorporated into free pyruvate. The same enzyme preparations do, however, catalyze the reduction of pyruvate to lactate by $DPNH^2$ in the absence of malate. The authors suggest that the "malic" enzyme and lactic dehydrogenase may be bound as a complex and that enzymatically formed pyruvate bound to the enzyme is reduced to lactate faster than it equilibrates with pyruvate in solution.

Another analogous situation exists in the synthesis or oxidation of butyrate by cell-free extracts of *Clostridium kluyveri* wherein it was found (9) that added acetoacetate is not in equilibrium with the enzymatically formed acetoacetate intermediate produced in the oxidation of labeled butyrate or in the synthesis of butyrate from labeled acetate and acetyl-P^2. Under appropriate conditions, however (in the absence of orthophosphate), free acetoacetate is formed (10) as an end product of butyrate oxidation. Recent experiments in the author's (E.R.S.) laboratory indicate that acetoacetyl coenzyme A is the normal intermediate which, in the presence of orthophosphate, does not hydrolyze to acetoacetate and CoA^2 but is preferentially converted to acetyl-P, acetate, and CoA.

During the oxidation of 2-C^{14}-acetate by cell suspensions of acetate adapted *Escherichia coli* (11) or *A. aerogenes* (12), isotopic carbon was incorporated into the methylene carbons of carrier succinate and malate, but

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[4] Personal communication.

not (or only slightly) into carrier α-ketoglutarate. Since the lack of labeling in α-ketoglutarate could not be attributed to impermeability, it was concluded that α-ketoglutarate is not an intermediate in the synthesis of succinate but that succinate may be formed directly by way of a Thunberg-type condensation of two molecules of acetate.

The above observations have been confirmed by Krampitz and associates.[4] However in other very beautiful isotope studies (13) they have demonstrated that the Thunberg cycle does not function in *E. coli* to a significant extent. They studied the oxidation of acetate anaerobically with fumarate as the oxidant and obtained as did Krebs (14), the following reaction:

$$1 \text{ acetate} + 4 \text{ fumarate} + 2H_2O \rightarrow 4 \text{ succinate} + 2CO_2. \qquad 2.$$

With 1-C^{14}-acetate it was found (15) that the succinate formed contained essentially all of the isotope; very little was incorporated into the CO_2. To determine whether the conversion of acetate to succinate occurs by way of the TCA cycle or a Thunberg condensation an experiment was performed using highly labeled 2-C^{13}-acetate (13). The labeled succinate formed was converted to ethylene and the mass composition of the ethylene was determined in a spectrometer. A mixture of ethylene with masses of 29 and 28 was found; no ethylene of mass 30 could be detected. Since a methyl to methyl condensation of acetate (i.e., a Thunberg-type condensation) would give rise to ethylene of mass 30, these results preclude such a mechanism under the experimental conditions used. The results are, however, compatible with a fixation of acetate carbon into succinate via the TCA cycle.

In view of the results presented in the above section it would appear premature to discard, at least in principle, previously established pathways on the basis of isotope experiments in which a lack of expected equilibration with added carriers is observed. While the nonequilibration experiments cast doubt on the pathway, per se, there is now evidence that the enzymatically produced intermediates may exist in the form of enzyme-substrate or coenzyme-substrate complexes. The extent of isotopic equilibration in such instances will depend upon the relative rates of dissociation of such complexes as compared to the rate of further metabolism. Variations in these respective rates might account for the differences observed from organism to organism and between the various intermediates (12) within a given organism.

Experiments with cell suspensions of *A. aerogenes* (4, 12) showing that the oxidation of labeled acetate in the presence of added TCA cycle compounds results in a dilution of the acetate with unlabeled carbon have been interpreted (4, 5, 12) as contraindicating the operation of the conventional TCA cycle, since by the latter cycle no acetate is formed. It should be pointed out, however, that acetate might be formed from the added TCA cycle intermediates by reactions unrelated to the immediate mechanism of acetate oxidation, for example, from oxaloacetate via pyruvate.

Cheldelin and associates (16, 17, 18) have shown that aspartate isolated from the proteins of washed glucose-grown cells of *Saccharomyces cerevistae*

after incubation with 1-C^{14}-acetate is labeled exclusively in the carboxyl groups. Although the latter carboxyl-labeling can arise through operation of the TCA cycle, these workers prefer to interpret their results as indicating that a methyl to methyl condensation of acetate occurs since, "during growth, when oxalacetate is being removed by conversion to aspartic acid, some other means must be available to supply C_4 acid" (18). However, this argument apparently does not apply to the interpretation of their results, since in an earlier paper (16) it was shown that under the experimental conditions used, with acetate as the sole carbon source, no significant growth occurs.

In previous simultaneous adaptation studies it was observed that acetate adapted cells of *Azotobacter agilis* (19) and *E. coli* (20) are able to oxidize acetate readily but will oxidize various other TCA cycle intermediates only after a pronounced lag period. These results were interpreted as indicating that the conventional TCA cycle may not operate as the major pathway of acetate oxidation in these organisms. Recent studies by Stone & Wilson (21), however, suggest that the observed lags with various TCA cycle intermediates is not a result of the absence of the respective oxidative enzymes but is probably associated with overcoming permeability difficulties. Thus, they observed that glucose grown cells of *Azotobacter vinelandii* oxidize the various TCA intermediates only after a lag and do not attack citrate. However, cell-free extracts of these organisms attack all substrates readily. For optimal rates of acetate oxidation, CoA, Mg^{++}, DPN^2, orthophosphate, and a catalytic amount of a TCA cycle compound had to be added. In other studies (22) these workers obtained additional support for the TCA cycle in *A. vinelandii* by showing that the oxidation of tagged acetate by cell-free extracts results in rapid fixation of isotope into citrate which is followed by its appearance into α-ketoglutarate and succinate. In summary it may be said that to date there appears to be no conclusive evidence disproving the TCA cycle as a major pathway of acetate oxidation in the microorganisms thus far examined.

Acetate activation.—Two mechanisms exist for the synthesis of acetyl CoA from ATP^2, acetate and CoA in microorganisms. They are as follows:

$$\text{I} \quad ATP + CoA \leftrightharpoons CoA\text{-}PP + AMP^2 \qquad \qquad 3.$$

$$CoA\text{-}PP^2 + \text{acetate} \rightleftarrows \text{acetyl CoA} + PP \qquad \qquad 4.$$

$$\text{Sum:} \quad ATP + CoA + \text{acetate} \leftrightharpoons \text{acetyl CoA} + AMP + PP \qquad 5.$$

and

$$\text{II} \quad ATP + \text{acetate} \leftrightharpoons \text{acetyl-P} + ADP^2 \qquad \qquad 6.$$

$$\text{Acetyl-P} + CoA \rightleftarrows \text{acetyl CoA} + Pi^2 \qquad \qquad 7.$$

$$\text{Sum:} \quad ATP + \text{acetate} + CoA \leftrightharpoons \text{acetyl CoA} + ADP + Pi \qquad 8.$$
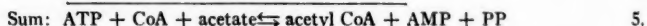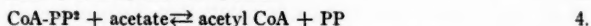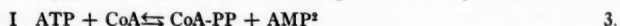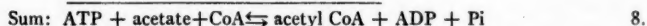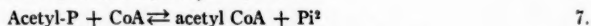
Evidence for the first mechanism, which also occurs in animals, has been obtained through the studies of Lipmann and his associates (23, 24, 25). Their results have been summarized by Lipmann (26). Using partially purified enzyme preparations from yeast extract they have established the stoichiometry for the over-all reaction 5 and have demonstrated its reversibility.

The occurrence of reaction 4 is implied from experiments showing that in the presence of the yeast enzyme fraction, acetyl CoA is decomposed by pyrophosphate. The CoA-PP presumed to be formed has not yet been isolated and characterized, nor has reaction 3, as such, been directly demonstrated.

The synthesis of acetyl CoA by reaction 8 occurs through the joint action of two enzymes that have been extensively purified. The enzyme catalyzing reaction 6 is widely distributed in bacteria and has been recently purified from extracts of *Streptococcus hemolyticus* by Korey.[4] The enzyme apparently catalyzes a direct transfer of the phosphoryl group from ATP to acetate and does not require CoA. The equilibrium constant of the reaction as determined by Korey at pH 7.3 is 77 favoring ATP formation.

Phosphotransacetylase, which catalyzes reaction 7 is also widely distributed in bacteria. This enzyme, which has been extensively purified (27, 28), was shown to catalyze a net synthesis of acetyl CoA from acetyl-P and CoA according to reaction 7 (29). The reaction can be followed spectrophotometrically (30) by measuring the change in light absorption associated with the formation of the thiol ester bond of acetyl CoA. From equilibrium measurements of reaction 7 it has been determined that the change in free energy associated with the conversion of acetyl-P to acetyl CoA is -2500 calories. From the equilibrium data obtained by Korey for reaction 6 it can be calculated that the change in free energy associated with the formation of acetyl-P from ATP and acetate is $+2600$ calories at pH 7.3 (28°C.). It follows, therefore, that the ΔF for reaction 8 would be close to zero.

Acetate synthesis.—*Clostridium thermoaceticum* ferments glucose with an almost quantitative yield of acetate (31). Barker & Kamen (32) demonstrated that in the presence of $C^{14}O_2$ the acetate formed is labeled in both carbon atoms to about the same extent and they suggested that the overall fermentation could be described as follows:

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 8H + 2CO_2 \qquad 9.$$

$$8H + 2C^{14}O_2 \rightarrow C^{14}H_3C^{14}OOH + 2H_2O \qquad 10.$$
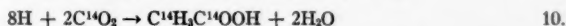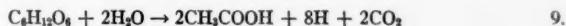
Accordingly, the acetate formed should be a mixture of labeled and unlabeled acetate in which one-third of the total acetate would be doubly labeled. This over-all mechanism is supported by results from the very ingenious experiments of Wood (33) who studied the fermentation of glucose in the presence of $C^{13}O_2$. The acetate produced was converted to ethylene and the relative abundance of $H_2C^{13}\!=\!C^{13}H_2$ (mass, 30) and $H_2C^{13}\!=\!C^{12}H_2$ (mass, 29) to $H_2C^{12}\!=\!C^{12}H_2$ (mass, 28) was determined. Part of the acetate was degraded to determine the distribution of C^{13} in the methyl and carboxyl carbons. The results show that 26 to 32 per cent of $C^{13}H_3C^{13}OOH$, 27 to 33 per cent of $C^{12}H_3C^{13}OOH$, 2.3 to 5 per cent of $C^{13}H_3C^{12}OOH$, and 30 to 42 per cent of $C^{12}H_3C^{12}OOH$ were formed. The formation of about 30 per cent $C^{12}H_3C^{13}OOH$ was explained by further experiments with C^{14}-acetate showing that during glucose fermentation, the carboxyl group, but not the methyl group of acetate equilibrates with CO_2.

In still other studies on the fermentation of 1-C^{14}-glucose and 3,4-C^{14}-glucose by *C. thermoaceticum*, Wood (34) obtained results suggesting that glucose may be fermented by a normal glycolytic mechanism leading to two C_3 fragments which in turn yield two molecules of acetate and $2CO_2$, followed by a conversion of the latter to another molecule of acetate. While these studies show that acetate is really formed by the condensation of two molecules of CO_2, the mechanism of this condensation is still obscure.

Propionic acid.—Using washed cell-suspensions obtained by differential sedimentation of rumen contents, Sijpesteign & Elsden (35) have shown that succinate is converted quantitatively to propionate and CO_2. These results are in accord with those obtained with pure cultures of *Viellonella gazogenes* (36) and *Propionibacterium pentosus* (37) and, when considered together with results of *in vivo* studies (35), indicate that the decarboxylation is a major pathway of succinate metabolism in the rumen of sheep. On the other hand, Johns (38) has found that cell suspensions of *Clostridium propionicum* do not produce CO_2 from succinate, malate, or fumarate and, from fluoride inhibition studies and tracer experiments, he has obtained evidence that propionate formation by *C. propionicum* does not involve the succinate pathway. In this organism propionate may possibly arise as follows: (38, 39) lactate $\xrightarrow{-H_2O}$ acrylate $\xrightarrow{+2H}$ propionate. A comparison of the isotope distribution patterns of the succinate and propionate formed in the fermentations of glucose-1-C^{14} and glucose-3,4-C^{14} by *Propionibacterium arabinosum* indicates that the propionate must arise from other sources in addition to succinate (40).

Butyrate oxidation.—The results of recent investigations on the oxidation of butyrate in bacteria have been thoroughly reviewed (41, 42, 43). In the presence of orthophosphate, extracts of *C. kluyveri* oxidize butyrate (44) according to the reaction:

$$\text{butyrate} + \text{orthophosphate} + O_2 \rightarrow \text{acetyl-P} + \text{acetate} + H_2O \qquad 11.$$
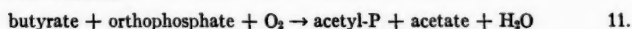
The report (44) that no oxidation occurs in the absence of orthophosphate was contradicted by the experiments of Kennedy & Barker (10) showing that butyrate is oxidized to acetoacetate under these conditions. These experimental differences appear to be reconciled by the demonstration that there is large variation in the ability of different dried cell-preparations to oxidize butyrate in the absence of orthophosphate (Stadtman[5]). With the discoveries in cell-free extracts of *C. kluyveri* of the enzymes phosphotransacetylase (27, 45, 46) catalyzing reaction 7, and a CoA transphorase (30, 45, 47) that catalyzes reaction 12

$$\text{acetyl CoA} + \text{butyrate} \leftrightarrows \text{butyryl CoA} + \text{acetate} \qquad 12.$$
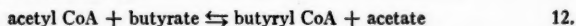
the mode of synthesis of butyryl CoA from acetyl-P, CoA and butyrate was elucidated.

[5] Unpublished data

The possibility that butyryl CoA is the immediate substrate for butyrate oxidation (41, 42) is supported by the discovery that synthetic (30) butyryl CoA is readily oxidized by dialyzed enzymes (Korkes & Stadtman[5]). Therefore, the need for acetyl-P to initiate butyrate oxidation (41, 44) may be attributable to the formation of butyryl CoA by reactions 7 and 12.

Based on the information available from studies on *C. kluyveri*, Barker (41) and Kennedy & Lehninger (42) have suggested that butyrate oxidation may be described by the following scheme:

```
                                    I
           ┌──────────► Acetyl CoA + butyrate ⇌ butyryl CoA + acetate
           │                                        II │↑ ±2H
           │                        VII                │
           │                      ± acetate            ▼
           │   Acetyl CoA + vinylacetate ⇌    vinylacetyl CoA
           │                                       III │↑  ± 2H, H₂O
           │                        VI                 │
           │                      ± acetate            ▼
           │   Acetyl CoA + acetoacetate ⇌    acetoacetyl CoA
           │                                        IV │↑  ± CoA ◄─┐
           │                                           ▼           │
           └─────────────────────────────────── 2 acetyl CoA      │
                                                  V │↑ ± Pi        │
                                                    ▼              │
                                             Acetyl - P + CoA ─────┘
```
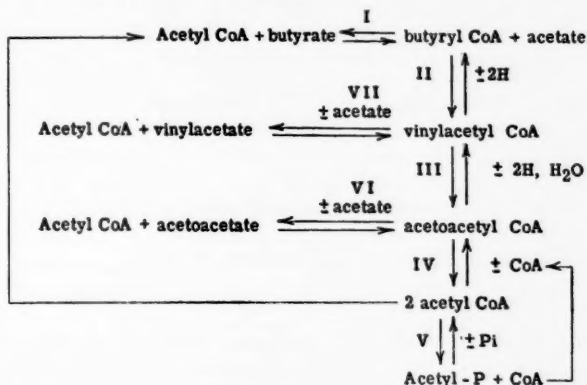
Thus, in the presence of orthophosphate (when reaction VI is not involved), the oxidation of butyrate is visualized as a cyclic process in which CoA and acetyl CoA have catalytic roles, and in which butyryl CoA, vinylacetyl CoA, and acetoacetyl CoA are intermediates. In the course of one complete cycle, one mole of butyrate is oxidized, and one mole of acetate (reaction I) and one mole of acetyl P (reaction V) are produced. Since only catalytic amounts of CoA and acetyl CoA are available, the continued regeneration of these substances is obligatory to the smooth operation of the cycle. This is accomplished through the combined actions of the two enzymes, β-ketothiolase and phosphotransacetylase. The β-ketothiolase catalyzes the thiolytic cleavage of acetoacetyl CoA by free CoA to form two molecules of acetyl CoA (48). Under the influence of phosphotransacetylase, one of these acetyl CoA molecules is decomposed with orthophosphate to form acetyl-P, thus regenerating CoA which is available for re-use in reaction IV. The other molecule of acetyl CoA derived from acetoacetyl CoA is used for the formation of butyryl CoA (reaction I) needed to start the cycle over again.

This scheme explains all of the observed facts insofar as the oxidation of butyrate to acetyl-P and acetate is concerned; however, it is not in complete accord with results obtained in the absence of orthophosphate when acetoacetate is produced. Thus the data presented by Kennedy & Barker

(10) and which have been confirmed by the author (E.R.S.[5]) show that the ratio of oxygen consumed:acetoacetate formed is about 2:1. According to the above scheme, however, the O_2 uptake:acetoacetate ratio should be 1:1. Furthermore, direct attempts to measure the formation of acetoacetyl CoA by reaction of acetyl CoA with acetoacetate (reaction VI) have been unsuccessful although reactions I and VII are readily demonstrated (49).

An alternative suggestion to explain the observed stoichiometry is as follows:

$$
\begin{array}{c}
\text{I}_\text{A} \\
\text{2 acetyl CoA} \; + \; \text{2 butyrate} \; \rightleftarrows \; \text{2 butyryl CoA} \; + \; \text{2 acetate} \\
\text{IV}_\text{A} \qquad\qquad \text{II}_\text{A} \\
+\text{CoA} \qquad\qquad\qquad\qquad \pm 8\text{H}, \, 2\text{H}_2\text{O} \\
\text{2 acetoacetyl CoA} \\
\text{III}_\text{A} \; \Big| \; +\text{H}_2\text{O} \\
\text{CoA} \; + \; \text{acetoacetate} \; + \; \text{H}^+
\end{array}
$$

The over-all reaction by such a mechanism would be

$$2 \text{ butyrate} + 3\text{H}_2\text{O} \xrightarrow{\;-8\text{H}\;} \text{acetoacetate} + 2 \text{ acetate} + \text{H}^+ \qquad\qquad 13.$$

Extensive butyrate oxidation by the latter scheme requires that reaction IV A is faster than reaction III A. This is a reasonable assumption since the rate of butyrate oxidation in the absence of orthophosphate is always slower than that in the presence of orthophosphate (10). Also, according to this scheme, a deficiency in reaction III A would result in the accumulation of acetoacetyl CoA, and the reaction would stop when all the CoA is used up. The latter situation appears to be realized in experiments with an enzyme preparation of *C. kluyveri* that catalyzes butyrate oxidation readily in the presence of orthophosphate and catalytic amounts of CoA but almost not at all in the absence of orthophosphate (and is therefore presumably deficient in acetoacetyl CoA hydrolyase catalyzing step III A). With this enzyme preparation and with the substrate amounts of CoA the oxidation of butyrate proceeds almost stoichiometrically (as measured by O_2 uptake) with respect to the CoA added, and an acetoacetyl ester (identified as the pyrazolone derivative) is formed (Korkes & Stadtman)[5]. Another consequence of reaction 13 is that one mole of acetate would be formed for each mole of butyrate oxidized and thus, no net decrease in volatile acids would occur.

On the other hand, in the scheme postulated by Kennedy & Barker (10), butyrate is oxidized quantitatively to acetoacetate, with acetate having a catalytic role. According to their scheme, there should be a net decrease in volatile acids equivalent to the acetoacetate formed. Although a net decrease in volatile acids was observed during the oxidation of butyrate and caproate, this decrease was much less than would be predicted from their scheme. In recent experiments by the author (E.R.S.[5]), dialyzed extracts of *C. kluyveri* were allowed to oxidize butyrate to completion in the absence of Pi. Under

these conditions, 0.63 mole of acetoacetate and 0.75 mole of acetate were produced per mole of butyrate oxidized. These data are in fair agreement with reaction 13. Since slightly more than one-half mole of acetoacetate is often formed per mole of butyrate oxidized, it may be that both of the above reaction mechanisms are operative with the latter one predominating.

The results of Cohen and associates with cell-suspensions of *Clostridium saccharobutyricum* and *Clostridium acetobutylicum* were recently reviewed (43). The pattern of butyrate oxidation in these organisms is similar, if not identical, with that observed in *C. kluyveri*. Butyrate oxidation by *Streptococcus mitis* leads to the formation of two moles of acetate. Valerate is also oxidized slightly but other fatty acids are not attacked (50).

Murkherjee (51, 52) has shown that growing cultures and cell-suspensions of *Aspergillus niger* oxidize butyrate, β-hydroxybutyrate and crotonate to acetoacetate. With butyrate, trace amounts of β-hydroxybutyrate and crotonate are also formed. Crotonate appears to be excluded as a free intermediate in butyrate oxidation since HCN (1 mg./ml.) inhibits the oxidation of crotonate completely but inhibits butyrate oxidation only 47 per cent and is without effect on β-hydroxybutyrate oxidation (52). In the presence of HCN, a greater accumulation of crotonate occurs.

The results are compatible with the following over-all scheme:

$$\text{butyrate}\begin{array}{c} \xrightarrow{\text{I}} \text{crotonate} \\ \text{II} \quad \updownarrow \\ \xrightarrow{} \beta\text{-hydroxybutyrate} \longrightarrow \text{acetoacetate} \end{array}$$
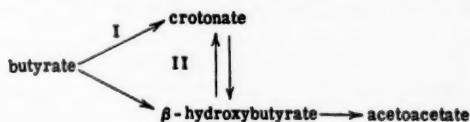
Step II is presumably inhibited by cyanide.

The accumulation of crotonate from butyrate is inhibited by the addition of β-hydroxybutyrate. The explanation offered by Murkherjee (52) to account for the latter observation is that crotonate and β-hydroxybutyrate are in equilibrium with each other (see scheme), and that the addition of β-hydroxybutyrate causes a shift in the equilibrium to the side of β-hydroxybutyrate with a consequent decrease in concentration of crotonate. This interpretation is not thermodynamically sound. It appears more likely that β-hydroxybutyrate inhibits the enzyme catalyzing the oxidation of butyrate to crotonate (step I).

Higher fatty acids.—In previous studies, Silliker & Rittenberg (53) have demonstrated that cell suspensions of *Serratia marcescens* oxidize saturated fatty acids with 2 to 14 carbon atoms. Oxidation by glucose-grown cells occurs only after a lag period indicating that adaptive enzymes might be involved. To obtain further information on this point, these workers have studied the effects of DNP[2] on fatty acid oxidation by so-called adapted and unadapted cells. It was found that DNP inhibits the oxidation of 2, 6, and 7 carbon fatty acids by both types of cells. It prevents adaptation to the oxidation of 8, 9, 11, and 13 carbon acids but not adaptation to 10, 12, and 14 carbon acids. Whereas in the absence of DNP, adaptation to any one fatty

acid causes simultaneous adaptation to all others (54), in the presence of DNP, the oxidation of 10, 12, or 14 carbon acids (completely to CO_2) does not cause simultaneous adaptation to fatty acids with fewer carbon atoms. Silliker & Rittenberg point out that these results appear to exclude free short chain acids as intermediates in the oxidation of the higher fatty acids but are consistent with the possibility that activated fatty acid derivatives are involved. As opposed to β-oxidation they seem to favor the idea that fatty acid oxidation in this organism proceeds by the multiple-alternate oxidation mechanism. Similar conclusions were reached from studies on the oxidation of omega-phenyl substituted fatty acids by *Pseudomonas fluorescens* and *Serratia marcescens* (55).

In view of the more recent work establishing acyl CoA derivatives as intermediates in butyrate oxidation by *C. kluyveri* (see above) and in the oxidation of fatty acids by animal enzyme preparations (48) it now seems more probable that the above results are a result of the occurrence of acyl CoA compounds as intermediates in fatty acid oxidation. The adaptive enzymes involved may be enzymes responsible for the activation of the free fatty acids rather than the oxidative enzymes as such. Variations in the DNP effect could be attributed to the existence of separate enzymes needed for the activation of fatty acids of different chain lengths. Such specificity has already been demonstrated. Thus the CoA transphorase system of *C. kluyveri* will not catalyze the activation of fatty acids with more than eight (possibly nine) carbon atoms (49), and from animal studies (56, 57) it appears that separate enzymes are involved in the synthesis of acetyl CoA by reactions 3 to 5 and in the synthesis of higher fatty acid-CoA derivatives by analogous reactions. It is hoped that the very interesting studies of Silliker & Rittenberg will be extended to the enzyme level where a more definitive answer to these questions can be obtained.

Webley & DeKock (58) have shown that cell suspensions of glucose-grown *Proactinomyces opacus* Jensen (*Nocardia opaca* Waksman & Hendrik) oxidize saturated fatty acids with 1 to 16 carbon atoms and various long chain hydrocarbons. As judged by the reduction of methylene blue, the supernatant solutions from "crushed cells" also catalyze the oxidation of hydrocarbons (fatty acids were not tested).

A series of papers has appeared showing the influence of various cultural conditions on the accumulation and composition of fat in *Fusarium lini* and *Fusarium lycopersici* (59 to 62). Since the experiments are of a preliminary nature, conclusions as to the mode of fat synthesis in these organisms must await more detailed studies.

PYRUVATE METABOLISM

Great advances have been made in the understanding of the biochemical mechanisms of pyruvate oxidation in microorganisms. This subject was reviewed by Ochoa (63) and more recently by Gunsalus (64).

Pyruvate oxidase factor.—Continuing their studies with *Streptococcus*

faecalis, O'Kane & Gunsalus (65) have shown that the POF[2] is needed specifically for the oxidation of pyruvate (66) or α-keto butyrate (67) by cell suspensions of organisms grown on completely synthetic media with glucose as the sole carbon source. POF exists naturally in a variety of soluble forms (68, 69) which differ with respect to solubility in organic solvents, polarity, and acid properties. One of these has been isolated as a highly purified crystalline solid (70) and was given the name α-lipoic acid. The purified product is capable of supporting growth of *Lactobacillus casei* in the absence of acetate (71); it is able to replace protogen for the growth of *Tetrahymena gleii* (72) and an unknown factor (B.R.) for the growth of *Butyribacterium rettgeri* (73). Evidence indicating the probable identity of these various factors has been summarized (73).

Independent investigations by two groups of workers (64, 74, 75 and 76 to 79) have led to the chemical synthesis and complete characterization of α-lipoic acid. The structural formula arrived at by these groups is as follows:

$$H_2-C-CH_2-C-(CH_2)_4COOH$$
$$\quad\; | \qquad\qquad\quad |$$
$$\quad\; S \text{——————} S$$
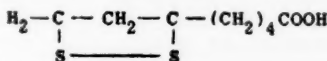
This compound is but one of several naturally occurring compounds having pyruvate oxidase activity, and it has been referred to variously as α-lipoic acid (75) DL-6-thiooctic acid (79) and Protogen A (77). The multiplicity of naturally occurring forms of α-lipoic acid (68) is explained by the existence of various mixed disulfide derivatives (69), the oxidized sulfoxide derivative (69, 74) which has been called Protogen B or β-lipoic acid (74, 77), and of various thiamine derivatives (80, 81) which are discussed below.

Lipothiamide.—Progress in elucidation of the chemical nature of what appears to be the coenzyme form of α-lipoic acid has resulted from the work of Reed & DeBusk (80 to 85). By ultraviolet irradiation of *E. coli* they have obtained a mutant strain that cannot grow on free α-lipoic acid but which will grow on certain more complex derivatives of α-lipoic acid that are present in extracts of various tissues and microorganisms. They report that α-lipoic acid "conjugates" that will support growth of the mutant can be prepared chemically by heating a mixture of α-lipoic acid with thiamine *in vacuo* or by the condensation of α-lipoyl chloride with thiamine monophosphate, thiamine pyrophosphate, or 2-methyl-5-ethoxymethyl-6-amino pyrimidine. Active compound formation with the latter substance is of particular interest since it precludes the involvement of the thiazole moiety of thiamine in the condensing reaction. Moreover, it indicates that the *E. coli* mutant may possess the ability to synthesize the thiazole moiety of thiamine and to condense this moiety with the conjugated pyrimidine.

Since the synthetic "conjugates" give negative thiochrome and positive azo tests it is concluded that the conjugates are formed by amide linkage between α-lipoic acid and the pyrimidine moiety of thiamine. Lipothiamide, lipothiamide monophosphate, and lipothiamide pyrophosphate would be

the corresponding derivatives of thiamine, thiamine monophosphate, and thiamine pyrophosphate respectively. Two active lipoic acid conjugates have also been prepared biosynthetically by incubation of washed cell suspensions of *S. lactis*, *S. faecalis*, or wild type *E. coli* with α-lipoic acid and thiamine (85). As judged by their chromatographic behavior on paper, and biological activity (i.e., the ability to support growth of the *E. coli* mutant) these biosynthetic products appear to be identical with naturally occurring substances isolated from buffer extracts of acetone-dried Fleischman's yeast, and with those produced chemically by the condensation of α-lipoyl chloride with thiamine and thiamine monophosphate. The authors concluded that the *E. coli* mutant does not possess the ability to condense thiamine and α-lipoic acid.

Enzyme studies.—Korkes et al. (86) made the important discovery that extracts of *E. coli* can be separated into two protein fractions, A and B. which, together with PTA[2], lactic dehydrogenase and the coenzymes, DPN, CoA and TPP[2] and Mg^{++}, are required for the dismutation of pyruvate to CO_2, acetyl-P and lactate. The additional requirement of this system for α-lipoic acid was demonstrated by Gunsalus et al. (64, 66) in their studies with POF-deficient cells of *S. faecalis*. Repeating the above fractionation studies with extracts of wild type *E. coli*, Reed & DeBusk (83) have confirmed the findings of Korkes et al.; however, they report that the corresponding A and B fractions obtained from the "conjugate"-requiring *E. coli* mutant will not catalyze the dismutation of pyruvate unless lipothiamide pyrophosphate is substituted for TPP.

A cross examination of the A and B fractions obtained from the wild type (fractions A_w and B_w) and the mutant (fractions A_m and B_m) showed that TPP was active in a system composed of A_m plus B_w but was not active in the system, A_w plus B_m. In still other experiments, it was shown that preincubation of B_w with TPP, followed by boiling to destroy the enzymes, yields a preparation, which together with fraction A_m plus CoA, DPN, PTA, and lactic dehydrogenase will cause the dismutation of pyruvate. The results have been interpreted as indicating that the B fraction contains an enzyme that catalyzes the synthesis of lipothiamide pyrophosphate from α-lipoic acid (present in the B fraction) and the added TPP. The preliminary reports of these very interesting investigations are only fragmentary in nature and a more critical evaluation must await further details. Nevertheless, several considerations are worthy of comment.[6] (a) In the presumed formation of lipothiamide pyrophosphate by fraction B_w, no α-lipoic acid is added (only TPP is added), and it is assumed that α-lipoic acid is present, bound to protein in fraction B. However, Gunsalus and associates[4] have found that whereas fraction A contains appreciable amounts of α-lipoic acid, there is very little in fraction B. (b) In the test system used by Reed &

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[6] These considerations are valid only if fractions A and B of Reed & DeBusk are identical with fractions A and B of Korkes et al. and of Gunsalus and associates.

DeBusk, PTA must be added. The PTA preparation used by them was a crude extract of *C. kluyveri* which has been shown to contain some B enzyme activity (Korkes).[4] (*c*) Gunsalus has shown (64) that with ferricyanide as the electron acceptor, fraction A catalyzes the oxidation of pyruvate to acetate and CO_2. This oxidation requires TPP. Neither fraction B nor CoA are needed. It follows, therefore, that if fraction B is needed to synthesize lipothiamide pyrophosphate, then the latter coenzyme is not specifically required for the ferricyanide coupled oxidation. Yet α-lipoic acid or a suitable derivative is apparently required since POF-deficient cells of *S. faecalis* will not catalyze the ferricyanide coupled reaction unless α-lipoic acid is added (64).

The last observations are of additional interest since they indicate the possibility that α-lipoic acid may be involved in electron transport between pyruvate and DPN. Seaman (87) on the other hand has observed that pyruvate oxidase preparations obtained from *Tetrahymena gleii* catalyze the reduction of 2,6-dichlorophenol indophenol in the absence of added protogen, whereas the formation of acetyl groups from pyruvate (as measured by hydroxamic acid formation) does not occur unless protogen is added. He concluded that protogen is not implicated in electron transport but is concerned with acetyl transfer. This interpretation is open to question, however, since no quantitative data were given for the dye reduction experiments nor were the products of oxidation established. The possibility remains that pyruvate may also be oxidized by a nonprotogen dependent mechanism, viz., by decarboxylation to free acetaldehyde and oxidation of the latter to acetate, or alternatively, to diacetyl via acetoin.

Nisman & Mager (88) report that extracts of *C. saccharobutyricum* catalyze the aerobic oxidation of pyruvate only if CoA and orthophosphate are present. The phosphate requirement is not readily understood since acetyl-P does not accumulate nor is added synthetic acetyl-P decomposed. Aceto-acetate which is formed accounts for only about one-half of the pyruvate oxidized.

In contrast to the other bacteria examined, pyruvate oxidation in *Proteus vulgaris* apparently proceeds in the absence of CoA or orthophosphate (89). Moyed & O'Kane (89, 90) have succeeded in separating cell-free extracts of this organism into two protein fractions both of which are needed for the oxidation of pyruvate to CO_2 and acetate. Pyruvate oxidation is mediated by a soluble cocarboxylase-dependent dehydrogenase present in fraction 2 which is coupled with an auto-oxidizable cytochrome system present in a particulate fraction (fraction 1). The dehydrogenase activity is completely inhibited by $10^{-5}M$ $HgCl_2$. As judged by the very low concentrations of flavin-adenine-dinucleotide and POF in the purified fractions, and also, by the fact that arsenite (0.03M) (which inhibits pyruvate oxidation by *S. faecalis* completely) is without effect on this system, it is concluded that these cofactors are not involved in pyruvate oxidation by *P. vulgaris*.

Acetoin formation.—It has been firmly established that acetoin formation

in microorganisms can occur by at least two basically different mechanisms. Juni (91) demonstrated that the conversion of pyruvate to acetoin in extracts of *A. aerogenes* can occur by the following successive reactions:

$$2 \text{ pyruvate} \rightarrow \alpha\text{-acetolactate} + CO_2 \qquad 14.$$
$$\alpha\text{-acetolactate} \rightarrow \text{acetoin} + CO_2 \qquad 15.$$
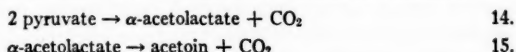
The enzymes catalyzing these reactions were separated from each other and the stoichiometry of the individual reactions was established (91). The formation of α-acetolactate from pyruvate was shown to require cocarboxylase. Acetone powders or cell-free extracts of various other bacteria, i.e., *Bacillus subtilis. Micrococcus pyogenes* var. *aureus*, and *S. marcescens* catalyze the decarboxylation of α-acetolactate and may therefore also form acetoin by the above mechanism. On the other hand, cell-free extracts of bacteria that do not produce acetoin from pyruvate, e.g., *E. coli, M. lysodeikticus*, and *Rhodospirillum rubrum*, do not catalyze the decarboxylation of α-acetolactate. Gunsalus (64) obtained evidence that α-lipoic acid is not essential for the conversion of pyruvate to α-acetolactate by *S. faecalis*. In contrast to the above mechanism, acetoin formation in other organisms occurs by a reaction between pyruvate and acetaldehyde.

Juni (92) points out that this conversion can be visualized as occurring by two different mechanisms. (A) The pyruvate may be converted to nascent acetaldehyde plus CO_2, and the nascent acetaldehyde may then condense with ordinary acetaldehyde in a benzoin-type condensation in which the double bonded oxygen of the added aldehyde is converted to the hydroxyl group of the ketol. (B) The acetaldehyde may condense directly with the ketogroup of the pyruvate to form α-acetolactate which may be decarboxylated to form acetoin (reaction 15). In mechanism A, the carbonyl group of the acetoin will be derived from the pyruvate carbonyl, but in mechanism B the acetoin carbonyl is derived from the acetaldehyde. Degradation of the acetoin produced from unlabeled pyruvate and 2-C^{14}-acetaldehyde by an enzyme fraction obtained from yeast and, also, of the acetoin produced from 2-C^{14}-pyruvate and unlabeled acetaldehyde by a pig heart enzyme preparation indicated that in both instances the acetoin was formed by reaction mechanism A (92). The results exclude α-acetolactate as an intermediate in these reactions.

Happold & Spencer (93) have observed that acetate, but not acetaldehyde, causes a stimulation in CO_2 and acetoin formation from pyruvate by protein fractions of *A. aerogenes*, and they suggest that acetoin formation may proceed by a mechanism in which an active aldehyde derivative (enzyme-aldehyde complex), obtained by decarboxylation of pyruvate, may react with free acetate to form diacetyl which is then reduced to acetoin. Although such a mechanism has not been excluded, it appears unlikely in view of the definitive experiments of Juni showing that α-acetolactate is an intermediate in the acetoin synthesis by this organism.

Cell suspensions of *P. fluorescens* and *P. aeruginosa* grown in the presence

of 2,3-butanediol are able to oxidize this substance to CO_2 and water (94). Diacetyl and acetoin were also identified as products of oxidation. Oxidation of the diol in the presence of 1 per cent sodium bisulfite leads to the accumulation of acetaldehyde. In the absence of oxygen, one mole of acid is formed per mole of diacetyl decomposed. Acetic acid was identified as an end product. From these results Sebek & Randles (94) postulate the following mechanism of butanediol oxidation.

$$\text{butanediol} \xrightarrow{-2H} \text{acetoin} \xrightarrow{-2H} \text{diacetyl} \longrightarrow \text{acetaldehyde} + \text{acetate} \qquad 16.$$
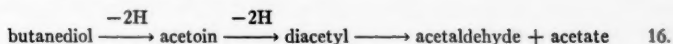
It remains to be seen if such a sequence of reactions can be demonstrated by a more critical enzymatic analysis of this system.

In studies with cell suspensions of *L. arabinosus*, Nossal (95) has noted that the conversion of pyruvate to acetoin and CO_2 is markedly stimulated by additions of KCl and trace amounts of glucose. Aging of cells in the absence of glucose results in a loss of their ability to decompose pyruvate anaerobically. In view of the fact that McIlwain (96) has noted that DPN is readily decomposed by cell suspensions of many bacteria and that glucose in some instances retards this process, Korkes[4] has suggested that the requirement for glucose and K^+ may be associated with the generation of energy needed for resynthesis of coenzymes involved in pyruvate decomposition.

Acetaldehyde oxidation.—Several different kinds of aldehyde-oxidizing enzymes have been demonstrated in microorganisms. Seegmiller (97) reported the 80-fold purification of an aldehyde dehydrogenase from Fleischman's bakers' yeast that catalyzes the oxidation of acetaldehyde to acetate by triphosphopyridine nucleotide (TPN). The enzyme was activated by various divalent ions and did not require CoA. This enzyme is therefore different from the highly purified aldehyde dehydrogenase isolated from yeast by Black (98) which requires K^+ and cysteine for activity and which can use either DPN or TPN[2] as electron acceptors.

In contrast to the yeast enzymes, the aldehyde dehydrogenase purified from extracts of *C. kluyveri* (45, 99) was found to catalyze the reversible reaction

$$\text{acetaldehyde} + \text{DPN}^+ + \text{CoA} \rightleftharpoons \text{acetyl CoA} + \text{DPNH} + \text{H}^+ \qquad 17.$$
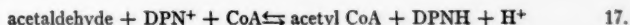
Although either DPN or TPN stimulated acetaldehyde oxidation in crude extracts of aged cell suspensions, the purified enzyme was DPN specific.

Studies with POF-deficient cell supensions of *S. faecalis* (100) indicate that POF is not required for the oxidation of acetaldehyde or ethanol to acetate.

C₁ COMPOUNDS

Formate and formaldehyde metabolism.—A more general role of formate or formaldehyde derivatives as intermediates in the metabolism of microorganisms has been emphasized by recent tracer studies. Jefferson *et al.* (101)

have shown that the fermentation of glucose in the presence of $HC^{14}OOH$ by cell suspensions of *Rhizopus MX* results in the formation of ethanol, lactate, and fumarate with relatively high specific labeling in the methyl and methylene carbon atoms. A similar distribution of isotope in lactate and malate was noted earlier when cell suspensions of *Rhizopus MX* were allowed to oxidize glucose in the presence of $2\text{-}C^{14}$-labeled ethanol (102). The fumarate formed when $2\text{-}C^{14}$-ethanol is oxidized by *Rhizopus nigricans* is almost exclusively labeled in the methylene carbons (103). From the latter results it was concluded[7] that C_4-dicarboxylic acids are formed by a direct $C_2 + C_2$ Thunberg type condensation (103). However, in view of the above mentioned results with labeled formate, another explanation is now suggested (101). The incorporation of formate carbon into the methyl and methylene carbons of ethanol, lactate, and fumarate could occur as follows:
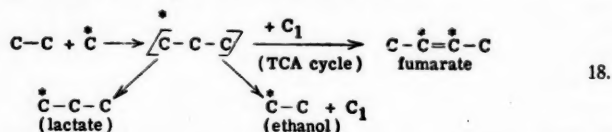
The unlabeled C_1 plus C_2 compounds ($C—C$ and C_1) are derived from glucose and the labeled compound (C^*) is formate or a suitable derivative. It has been pointed out (101) that by an established mechanism (105) the reaction of unlabeled glycine ($C—C$) with labeled formate (C^*) will give serine ($C^*—C—C$) containing an isotope distribution of the type postulated in the above scheme. It is, therefore, suggested (101) that the latter reaction may possibly be implicated in the mold fermentations. That this formate-fixing reaction may be of more general importance, is indicated by the further discovery (106) that ethanol produced anaerobically from glucose in the presence of $HC^{14}OOH$ by *Fusarium vasi-infectum*, *Aspergillus niger*, and *Saccharomyces cerevisiae* is labeled almost exclusively in the methyl group.

The distribution of isotope in citrate and malate formed during the oxidation of sucrose in the presence of $C^{14}O_2$, $HC^{14}OOH$ or $C^{14}H_3OH$ by cell suspensions of *A. niger* is consistent with the view that formate is converted to $C^{14}O_2$ which enters the TCA cycle via the Wood-Werkman reaction (107). Methanol was not oxidized appreciably to CO_2 and was not utilized appreciably for citrate synthesis.

Formic hydrogenlyase.—Recent investigations on the formic hydrogenlyase system which is responsible for the over-all reaction

$$HCOOH \rightleftarrows CO_2 + H_2 \qquad 19.$$

have been adequately reviewed by Gest (108). Results obtained by him (108,

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[7] This conclusion was questioned by Utter & Wood (104), who pointed out that similar labeling of fumarate from $2\text{-}C^{11}$-ethanol can be explained by an alternative mechanism.

109) in studies with *E. coli* show that this reaction is extremely complicated and appears to be catalyzed by the combined action of formic dehydrogenase, hydrogenase, and possibly other enzymes.

The involvement of formic dehydrogenase and hydrogenase is inferred from experiments showing that whereas little or no hydrogenlyase activity is present in hydrogenase preparations (obtained by appropriate dilution of soluble extracts of anaerobically grown cells), or in hydrogenase-free formic dehydrogenase preparations (obtained as particulate fractions from aerobically grown cells), good hydrogenlyase activity is obtained by a mixture of the two preparations.

Other studies indicate that Fe^{++} or Mn^{++} (110), cocarboxylase, diacetyl, or pyruvate (or a derivative of these compounds), and orthophosphate are needed for optimal hydrogenlyase activity (108). No requirement for CoA and various other coenzymes could be detected; DPN and TPN caused almost complete inhibition. In view of the multiplicity of cofactors required and the rather complex nature of the enzyme fractions used, it is still too early to decide with certainty if formic dehydrogenase and hydrogenase are definitely implicated in the hydrogenlyase system, much less to speculate on the type of reactions involved.

During the fermentation of various substrates by *Propionibacterium arabinosum*, formaldehyde is incorporated into every position of the propionic acid which is formed (111). In other studies (112) with $HC^{14}HO$ it was found that the ethylene carbons of the succinate formed have a specific activity almost twice that of the propionate ethyl carbons.

During the fermentation of glucose in the presence of dimedon the formaldehyde produced as an intermediate in the fermentation of glucose can be trapped as the formaldehyde-dimedon derivative. From specific activity measurements of formaldehyde accumulating in fermentations of glucose-1-C^{14} and glucose-3,4-C^{14}, it was shown that only 2.3 per cent and 23 per cent of the formaldehyde is derived from the number 1 and 3,4, carbons respectively. Thus the 2,5, or 6 carbons of glucose are the major sources of formaldehyde. As pointed out by Leaver (112), since formaldehyde is derived mainly from the number 1 carbon of glycerol-1-C^{14} (113), it seems probable that the number 6 carbon of glucose is the major source of formaldehyde in the above experiments.

Pine & Barker (114) have observed that when *B. rettgeri* is grown in a medium containing glucose or lactate and $HC^{14}HO$, isotopic carbon is incorporated into all carbon atoms of the fermentation products, lactate, acetate, and CO_2. An extensive incorporation into the methyl group of acetate and of lactate is in contrast to results obtained in comparable experiments with $C^{14}O_2$ and $HC^{14}OOH$ showing that these compounds are not appreciably incorporated into the methyl carbons. This indicates that the latter compounds are not obligatory intermediates in the formaldehyde reactions of this organism.

Nitrogen Compounds

Arginine metabolism.—Whereas in higher animals arginine is hydrolyzed to urea and ornithine by liver arginase, a number of microorganisms contain an arginine dihydrolase system that catalyzes the conversion of arginine to ornithine without concomitant urea formation (115 to 120). On the basis of present evidence the reaction can be written in two steps:

$$\text{arginine} \rightarrow \text{citrulline} + NH_3 \qquad\qquad 20.$$

$$\text{citrulline} \rightarrow \text{ornithine} + CO_2 + NH_3 \qquad\qquad 21.$$

Cell-free extracts of *S. faecalis* (116, 117, 119), *S. lactis* (120), and *Clostridium perfringens* (118) catalyze reaction 20 by a system that has been termed arginine desimidase (121) or metarginase (115). No cofactor requirements for this reaction can be demonstrated after dialysis.

The subsequent conversion of citrulline to ornithine by reaction 21 is catalyzed by a system termed citrulline ureidase (122) or citrullinase (115) present in extracts of *S. faecalis.* Inorganic phosphate, AMP (or ATP) and a divalent metal such as Mn^{++} or Mg^{++} are required for activity with these extracts [Slade (122)].

Superficially, these reactions appear to resemble those of the mammalian urea cycle leading to the synthesis of arginine from ornithine operating in reverse. However, it is evident that the bacterial degradative pathway is a complex one and must be studied in its component parts before the mechanism can be understood well enough to draw comparisons.

Evidence more suggestive of the basic similarity of arginine metabolism in various phyla comes from the finding by Walker (123) and by Davision & Elliott (124) that the compound, arginosuccinic acid, can be isolated from a wide variety of tissues, including dried cells of *Chlorella* and extracts of yeast and *Proteus vulgaris,* after incubation with arginine and fumarate. This compound has been shown by Ratner (125, 126, 127) to be the intermediate that is formed from citrulline and aspartate and is reversibly split to arginine and fumarate in the mammalian liver system.

$$\text{Citrulline} + \text{aspartate} + \text{ATP} \rightarrow \text{arginosuccinate} + \text{ADP} + \text{Pi} \qquad 22.$$

$$\text{arginine} + \text{fumarate} \rightleftarrows \text{arginosuccinate} \qquad\qquad 23.$$
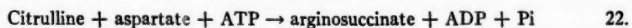
Preliminary experiments show that enzymes catalyzing these reactions are also present in yeast (127). Certain species of *Brucella* (128), of *M. pyogenes* var. *aureus* (129), and of *Hemophilus pertussis* (130) may contain similar systems. In view of the recent experiments by Lieberman & Kornberg[4] showing that ureidosuccinate is converted enzymatically to orotic acid it is tempting to speculate on the possibility that there may be some biological mechanism for removing the ureidosuccinate moiety from arginosuccinate, thus furnishing a precursor for pyrimidine synthesis. The isolation from fresh yeast of a carbamido compound having the empirical formula $C_7H_{14}O_2N_2$ also suggests that a wider variety of these compounds may exist

as biological intermediates than is at present suspected [Fearon & Boggust (131)]

Strassman & Weinhouse (132) have studied the biosynthesis of arginine from C^{14}-labeled acetate, formate, glycine, and lactate in yeast. Their results are consistent with the view that these compounds, or suitable derivatives, enter the TCA cycle to form α-ketoglutarate which serves as a precursor of the 5-carbon chain of arginine. Formate and glycine presumably are converted to serine which in turn is converted to pyruvate and enters the TCA cycle; likewise lactate may enter the cycle via pyruvate, whereas acetate can enter directly. As in other systems, the guanido carbon arises by carbon dioxide fixation (133, 134).

Vogel (135) has isolated α-N-acetyl-L-ornithine from an E. coli mutant that accumulates this compound in the growth medium. The acetylated compound is readily used by another E. coli mutant that requires either arginine, citrulline, or ornithine. Active deacylase preparations were obtained from the latter mutant and also from wild type E. coli, but not from the mutant that accumulates the compound.

Acetylation of the α-amino group may serve as a device for keeping the intermediates in the glutamate to ornithine sequence of reactions in the open chain form. Thus, if glutamate is reduced to γ-glutamic-semialdehyde, the latter will undergo spontaneous ring closure unless the amino group is covered. Evidence in favor of this view has been obtained recently with other E. coli mutants (Vogel[4]).

Proline synthesis.—Employing bacterial mutants, Vogel & Davis (136) have isolated and identified a precursor in the biosynthesis of proline. The compound, Δ^1-pyrroline-5-carboxylic acid, has also been prepared synthetically. Biologically, it is presumed to be formed from glutamate via γ-glutamic semialdehyde which spontaneously condenses to form the pyrroline-ring compound. Reduction of the double bond then can yield proline. The accumulation of Δ^1-pyrroline-5-carboxylic acid by one proline-requiring E. coli mutant and the utilization of this compound in the absence of proline by other proline-requiring E. coli and Neurospora mutants is evidence for its intermediary role in proline biosynthesis.

Pyridoxal-phosphate dependent reactions.—An excellent summary of the chemical properties and enzymatic functions of pyridoxal phosphate was presented by Snell (137). Study of the nonenzymatic decarboxylations, deaminations, and racemizations of amino acids when heated with pyridoxal and metal ions has contributed much to the understanding of reaction mechanisms. In addition, the dehydration of serine and desulfhydration of cysteine have also been studied under similar conditions by Metzler & Snell (138). The products are pyruvic acid, ammonia and, in the case of cysteine, H_2S. The mechanism is believed to involve Schiff base formation with pyridoxal and the amino acid, which is then dehydrated or desulfhydrated and finally hydrolyzed to yield pyruvate and ammonia.

The D-serine dehydrase from E. coli [Metzler & Snell (139)] and from N. crassa [Yanofsky (140)] is a pyridoxal-phosphate dependent enzyme and

catalyzes the deamination of both D-serine and D-threonine, forming pyruvate and α-keto butyrate respectively. The L-serine and L-threonine deaminase of *Neursopora* [Reissig (141)] also requires pyridoxal-phosphate.

An enzyme partially purified from cysteine-adapted *Proteus vulgaris* cells was completely resolved from its coenzyme by treatment with cyanide (142). The enzyme that phosphorylates pyridoxal probably was present in the fraction, for upon the addition of pyridoxal and ATP there was liberation of H_2S from cysteine, and pyruvate accumulated. At high concentrations of cysteine, alanine rather than pyruvate was found as a product. Whether this is a result of the reduction of the postulated intermediate, amino acrylic acid, by another mole of cysteine, as the authors suggest, rather than hydrolysis to pyruvate followed by reamination cannot be decided on the basis of the information available.

Hurwitz (143) has purified an enzyme from bakers' yeast that phosphorylates pyridoxal with ATP to form pyridoxal-phosphate. A number of the properties of this enzyme were studied, and various structural analogues and derivatives of pyridoxal were tested as substrates. One compound, 2-ethyl-3-amino-4-ethoxymethyl-5-amino-methyl pyridine, competitively inhibited phosphorylation of pyridoxal with ATP. The tyrosine apodecarboxylase from *S. faecalis* R was used for assay purposes.

The phosphorylated analogues of pyridoxal that inhibited the tyrosine apodecarboxylase possessed a hydroxymethyl or methylamino group in the five position and had no substituent in the six position. Several 5,6-disubstituted compounds containing substituents such as the hydroxymethyl group were not inhibitory.

Hydroxylamine and semicarbazide are believed to act as inhibitors of pyridoxal-phosphate catalyzed reactions by reacting with the aldehyde group of pyridoxal-phosphate. In agreement with this hypothesis, Roberts (144) found that the inhibition by hydroxylamine of glutamic acid decarboxylase in extracts prepared from acetone powders of *E. coli* could be overcome by the addition of pyridoxal phosphate. These studies strongly suggest that the aldehyde group is necessary for formation of the postulated Schiff base intermediate.

Further studies have been made on the substrate specificity of various amino acid decarboxylases. Roberts (145) compared mouse brain and *E. coli* glutamic acid decarboxylases and found α-methyl glutamic acid to be a potent inhibitor for both.

Sourkes *et al.* (146, 147) compared the tyrosine decarboxylase from *S. faecalis* R with the mammalian DOPA[2] decarboxylase as to substrate specificity employing several DOPA isomers and derivatives. The 2,4 and 3,4 DOPA derivatives were rapidly decarboxylated by the bacterial enzyme. However, 3,4-dimethoxyphenylalanine was not attacked.

Attempts to inhibit lysine and tyrosine decarboxylases with homologous rabbit antibodies were reported by Howe & Treffers (148) and Happold & Ryden (149). Howe & Treffers used a partially purified *E. coli* lysine decar-

boxylase and obtained an antiserum that largely precipitated the enzyme and inhibited its activity on lysine. It did not inhibit glutamic decarboxylase under the same conditions. However, the antiserum prepared for the tyrosine decarboxylase (149) of *S. faecalis* did not precipitate this enzyme.

Pyridoxal-phosphate has been implicated as the coenzyme for a glutamic acid racemase studied by Narrod & Wood (150). The racemization of glutamic acid by dried cells of *L. arabinosus* 17-5 is stimulated 3-fold upon the addition of pyridoxal phosphate. Starting with either D-glutamate or L-glutamate, a racemic mixture is approached. Ayengar & Roberts (151) found only a 20 per cent conversion of either isomer in their studies with the same organism, but might also have obtained racemic mixtures had they allowed the reactions to continue longer or used more enzyme. Their failure to show a pyridoxal phosphate stimulation may be related to the fact that the experiments were conducted at pH 8.1 while those of Narrod & Wood were carried out at pH 6.8. In the serine and threonine dehydrase reactions [Metzler & Snell (139)] the observation has been made that cells grown at a higher pH or adjusted to pH 7.8 before drying exhibit greater deaminase activity, and no dependence on pyridoxal phosphate.

An interesting development in the study of transamination reactions is the observation by Gunsalus & Tonzetich (152) that an enzyme preparation from *E. coli* catalyzes the transfer of amino groups derived from various purines, cytosine, and pyridoxamine to α-keto glutarate. The possibility exists that such transaminations may be involved in purine and pyrimidine interconversions if it is found that the corresponding keto derivatives are products of the deaminated purines or pyrimidines. These could not, however, be identical with the products of hydrolytic deaminations (i.e., hypoxanthine from adenine or xanthine from guanine) because an oxidation step is not involved in this process whereas the transamination reaction involves the oxidation of the amino group donor.

Pyridoxamine can also act as an amino group donor in transamination reactions, but this reaction, too, requires pyridoxal phosphate. The reverse reaction with glutamate as amino donor and pyridoxal as amino acceptor also occurs. Further evidence for the broad scope of transaminase reactions was obtained by Meister (153) who found that a wide variety of amino acids including ethionine, methionine, and chloroacetyl derivatives of lysine and ornithine could donate their amino groups to α-keto glutarate when added to *Lactobacillus plantarum* extracts.

Some progress has been made in the fractionation of transaminase activities in *B. abortus* extracts [Altenbern & Housewright (154)] and in *E. coli* extracts [Rudman & Meister (155)]. A fraction was obtained from the *B. abortus* extracts that contained a pyridoxal-phosphate stimulated leucine-alanine transaminase system but not a glutamic acid-alanine system. Rudman & Meister separated two transaminase systems from wild type *E. coli* extracts by fractionation with calcium phosphate or alumina gels and ammonium sulfate. One of these catalyzed the formation of glutamate from

α-keto glutarate and valine, leucine, isoleucine, norvaline, or norleucine; the other from α-keto glutarate and tyrosine, phenylalanine, tryptophan, or aspartate. The amino acids of each group transaminated with each other as well as with glutamate but very slowly or not at all with those of the other group. Extracts were also made of an *E. coli* mutant that requires isoleucine for growth, fails to use the corresponding keto acid, and exhibits a secondary requirement for valine. Such extracts did not catalyze transamination between these amino acids and α-ketoglutarate, but did yield an enzyme fraction catalyzing an amino group transfer between alanine or α-amino butyrate and α-keto isovalerate to form valine. In fact, when equivalent amounts of alanine or α-amino butyrate were added to the growth medium, the mutant no longer showed a requirement for valine. These findings support the conclusion that the mutation involves a loss in ability to catalyze a transamination between isoleucine or valine and glutamate. The valine requirement is not absolute because there exists another mechanism for its synthesis from alanine or α-amino butyrate and α-keto isovalerate. The latter transaminase system was also found in an enzyme fraction from wild type *E. coli*. Thus both *B. abortus* and *E. coli* appear to possess alanine-linked transaminases as well as glutamate-linked systems.

As pointed out by Meister, a comparable situation may well exist in *Neurospora* mutants [Bonner (156)] which also show a double requirement for isoleucine and valine. Enzymatic studies on these mutants would be of special interest in view of the fact that this is a frequently cited example (157, 158) in support of the thesis that accumulation of a precursor as a result of a genetic block in the synthesis of a compound (in this case isoleucine) completely inhibits the synthesis of a related compound (here valine).

As to whether a single enzyme catalyzes the amino group transfer from both valine and isoleucine to α-keto glutarate as suggested by Rudman & Meister, in view of their inability to resolve these activities, and as would be required by the "one gene—one enzyme" theory, remains to be seen. Umbarger & Magasanik (159), on the other hand, interpret their kinetic studies on the isoleucine and valine to α-keto glutarate transaminase reactions catalyzed by dried *E. coli* cells as indicative of two separate enzymes. Their data, however, showing typical competitive inhibition between valine and isoleucine are equally in favor of a single enzyme system.

Methionine.—The reader is referred to a recent review by Cantoni (160) on the synthesis and transfer of the labile methyl group in which is discussed the central role of methionine in both methyl group and sulfur metabolism. Although much of the available information has accumulated from nutritional, isotope, and enzyme studies with animal systems, a number of important contributions have come out of studies with microorganisms. Schlenk and co-workers (161, 162, 163) have found that the accumulation of adenine thiomethyl riboside in a large variety of yeasts is dependent on the presence of L-methionine. Moreover, the nucleoside contains isotope when methyl-C^{14} or S^{35}-labeled methionine is used but not when α, β, or γ-C^{14}-

methionine is added (163). The precursor is considered to be a compound similar to, or perhaps identical with, Cantoni's "active methionine," now known to be methionyladenosine formed by the reaction of ATP with methionine. A cleavage of methionyladenosine is visualized in which the carboxyl, α, β, and γ carbon moiety of methionine gives rise to homoserine leaving the thiomethyl moiety on the compound as thiomethyl adenosine. Many attempts to show the involvement of this compound in the reverse reaction, i.e., methionine synthesis, have been unsuccessful (161). However, Dubnoff (164) has found that under certain conditions a methionine-requiring mutant can use thiomethyl adenosine as well as PAB[2] or the methyl donor, dimethyl-β-propiothetin, (165) for growth on homocysteine containing media.

Another widely distributed, naturally occurring methyl donor is the methionine analogue recently isolated as the halide salt by Shive et al. (166) from cabbage juice. It appears to be identical with synthetic 3-amino-3-carboxy-propyl-dimethyl-sulfonium (methionine methyl sulfonium). Both the naturally occurring and synthetic compounds overcome sulfanilamide toxicity for E. coli and allow growth of methionineless E. coli and Neurospora mutants in the absence of methionine or B_{12}.

Cell suspension studies with various E. coli mutants (167, 168) have demonstrated a serine requirement for methionine synthesis from homocysteine. With one strain, equimolar amounts of glycine were formed during the synthesis of methionine from serine and homocysteine. Although the hypothesis is advanced that cystathionine may be the intermediate in such a reaction (167, 169) and be reductively cleaved to methionine and glycine there is, as yet, no direct evidence to support this possibility.

Studies on strains of Pasteurella pestis (170) and E. coli (171) that require methionine for growth show that methionine does not satisfy the entire sulfur requirement of these organisms. Thus the degradative as well as synthetic pathways may be blocked in these mutants. Wild-type E. coli cells grown in the presence of S^{35}-labeled methionine also incorporate only traces of S^{35} into the other sulfur amino acids of the bacterial proteins (171). However, this apparent stability of methionine sulfur during growth of an organism able to synthesize methionine may merely reflect the predominantly anabolic processes under these conditions rather than the nonreversibility of the reactions.

The sulfur-containing products of methionine breakdown by two soil pseudomonads, using methionine as a sole source of C, N, and S, were identified as methyl mercaptan and dimethyl disulfide (172). No dimethyl sulfide was found.

Lysine.—Dewey & Work (173, 174) have found that DAP,[2] an amino acid occurring in E. coli and A. aerogenes, is decarboxylated to lysine by acetone-dried cells or by extracts of these organisms. Unless the extracts are fractionated, the final product is cadaverine formed by the action of a lysine decarboxylase that is also present. Three lysine-requiring E. coli mutants

which accumulate large amounts of DAP [Davis (175)], were found to contain no detectable amounts of DAP decarboxylase. This substantiates Davis' earlier conclusion (175), that the genetic block is one preventing the decarboxylation of DAP.

The information regarding earlier precursors of lysine or possible alternative pathways of synthesis is still very meager. Davis (175) suggests that there is some interrelationship between threonine and lysine biosynthesis in *E. coli* since certain of the DAP or lysine-requiring mutants accumulate threonine.

Strassman & Weinhouse (176) have degraded protein lysine isolated from *Torulopsis utilis* grown on methyl or carboxyl-labeled acetate. From carboxyl-labeled acetate, the observed distribution of isotope in lysine, starting from the carboxyl position is 360, 0, 7, -11, -11, 226; with methyl-labeled acetate the distribution starting from the same carbon is 18, 206, 121, 116, 116, 7. Strassman & Weinhouse point out that one possible explanation for the observed labeling would be the addition of a C_2 compound to α-keto glutarate to form a C_7 intermediate, which by means of a series of reactions analogous to the tricarboxylic acid cycle, might yield α-keto adipic acid. Amination and reduction of this compound would presumably yield lysine. While there is no direct evidence for the postulated series of reactions leading to α-keto adipic acid, studies with *Neurospora* mutants indicate that α-amino adipic acid is converted to lysine [Mitchell & Houlahan (177)].

The oxidation of C^{14}-carboxyl-labeled lysine by a purified L-amino acid oxidase from *N. crassa* yields a product that no longer reacts with ninhydrin but is still radioactive (178). The compound is quite likely the hydropicolinic acid formed by spontaneous ring closure of α-keto-ϵ-amino caproic acid, the keto acid formed by oxidative deamination of lysine.

Histidine.—*P. fluorescens* extracts (179, 180) degrade histidine to L-glutamic acid, formate, and 2 moles of ammonia (Reaction 26). Acetone powders of *Clostridium tetanomorphum* appear to catalyze similar reactions (181).

$$\text{histidine} \xrightarrow{\text{histidase}} \text{urocanic acid} + NH_3 \qquad\qquad 24.$$

$$\text{urocanic acid} \xrightarrow{\text{urocanase}} \text{intermediate (s)} \rightarrow \text{glutamic acid} + HCOOH + NH_3 \qquad 25.$$

$$\text{histidine} \rightarrow \text{glutamic acid} + 2NH_3 + HCOOH \qquad\qquad 26.$$
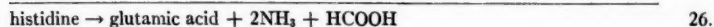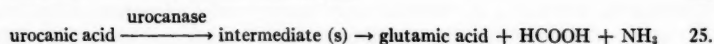
Carbon-2 of the imidazole ring is converted quantitatively to formate (182). N^{15}-studies show that one ammonia is derived from the α-amino nitrogen whereas the imidazole-N on the γ carbon becomes the amino group of glutamic acid (182). Thus the other ring N on the δ-carbon must also yield ammonia. The conversion of the α-amino N to ammonia is consistent with the view that the first step in the degradation involves a deamination yielding urocanic acid (Reaction 24). When C^{14}-labeled histidine was metabolized in the presence of unlabeled urocanate, the urocanate became labeled to the

degree expected if all the histidine was metabolized via this pathway (182).

A further degradation product of either histidine or urocanic acid (Reaction 25) has been isolated in crystalline form from a guinea pig liver system by Tabor *et al.* (183). Upon hydrolysis, the compound yields one mole of glutamic acid, one of ammonia, and one of formate. A similar, or possibly identical, compound is formed in the *Pseudomonas* system (Tabor & Mehler[4]). It now appears that the glutamic acid derivative isolated from the urine of folic acid deficient animals by Silverman *et al.* (184) is also the same compound (183). Similar compounds have previously been isolated from liver systems by Sera & Aihara (185) and Oyamada (186). However, the exact structure of these intermediates and the mechanism of their further degradation to glutamic acid, formate, and ammonia are not clear. Two of the possibilities that exist are: (*a*) either formate is split off first, leaving isoglutamine (185, 187) or (*b*) ammonia is removed with the formation of N-formyl glutamic acid. Some support for the latter possibility is given by the observation that *Pseudomonas* extracts rapidly deacylate N-formyl glutamic acid (179), whereas isoglutamine is not decomposed by these extracts (Mehler & Tabor[4]). The enzymatic steps in the histidine degradative system leading to the formation of free formate offer intriguing possibilities for study of a formyl coenzyme. This is especially interesting in view of the possible involvement of folic acid already mentioned.

Aromatic amino acids.—The status of our information on the origin of aromatic structures as deduced from isotope studies has been reviewed recently by Ehrensvard (188). Davis has considered the evidence obtained from studies with bacterial mutants concerning intermediates in the biosynthesis of phenylalanine, tyrosine, and tryptophan (189). The bacterial degradation of these compounds, with particular emphasis on tryptophan, is discussed in a review by Stanier (190) and briefly by Hayaishi (191). Therefore, only a few of the most recent contributions to the field will be considered here.

Isotope studies with yeast [Gilvarg & Bloch (192)] and *E. coli* mutants [Shigeura & Sprinson (193)] employing 1-C^{14}-glucose as substrate show that carbon-1 of hexose is incorporated to a very high degree into aromatic ring compounds. In certain *E. coli* mutants highly labeled shikimic acid accumulated. The yeast protein aromatic amino acids contained C^{14} in the β-carbon of the side chain and in ring carbons 2 or 6 or both. The specific activity of the ring carbons was only 50 to 70 per cent that of the glucose, whereas the specific activity of the residual glucose was unchanged. Thus a direct cyclization of glucose is excluded. A number of alternative mechanisms such as condensation of two isotopically equilibrated C_3 units, condensation of three C_2 units, or condensation of triose with tetrose to form heptulose also appear unlikely.

Studies by Davis *et al.* (194) with various *E. coli* mutants have revealed the identity of still another intermediate in the aromatic series. Thus, Davis' compound W (158) is now known to be 5-dehydroquinic acid, which upon

dehydration becomes 5-dehydro-shikimic acid. Reduction of the latter compound at the five position yields shikimic acid, a common precursor of the aromatic amino acids, PAB and POB.[2] As yet no open chain precursors of the cyclic intermediates involved in the biosynthesis of aromatic compounds have been identified.

There is a difference of opinion regarding the pathway of conversion of shikimic acid to the aromatic amino acids, PAB, and POB. Davis (195, 158) has found that a large number of *E. coli* mutants showing multiple requirements for these aromatic compounds (multiple aromatic auxotrophs) are blocked in the synthesis of precursors common to all. On the basis of his experiments he has postulated that the number of aromatic compounds required for growth is a function of the completeness of the block; thus with an increasing degree of blocking, there appears a progressive requirement for the following compounds in the order listed; tyrosine, phenylalanine, tryptophan, PAB, and POB. This conclusion is strengthened by the observation that a double mutant, blocked before and after 5-dehydro shikimic acid, is competitively inhibited by addition of 5-dehydro shikimic when grown on shikimic acid. In the presence of increasing inhibitor-substrate ratios, the aromatic amino acids, PAB, and POB must be added in the same order as above to allow growth of the mutant (158). From these and similar studies it is inferred that in *E. coli* the aromatic amino acids are not interconvertible but stem from a common precursor. If this proves to be the case, then *E. coli* differs in this respect from the rat which contains enzymes catalyzing the conversion of phenylalanine to tyrosine (196). Bergmann *et al.* (197) have interpreted their studies on sparing effects of aromatic amino acids on growth of *E. coli* mutants as indicating that these compounds are interconvertible. Supplementary enzyme studies on *E. coli* will be needed to determine whether one or both of the above conclusions is correct.

The quantitative interrelationship of tryptophan and nicotinic acid has been studied in tryptophanless *Neurospora* mutants employing N^{15}-labeled indole, anthranilic acid and tryptophan (198). For N^{15} measurements, quinolinic acid rather than nicotinic acid was isolated, since the nitrogens are apparently equivalent and the former compound is more readily obtained in suitable amounts. Comparison of the N^{15} content of the isolated tryptophan and quinolinic acid indicate that anthranilic acid-N and indole-N are converted into ring-N of tryptophan and nicotinic acid. Furthermore, in *Neurospora*, nicotinic acid-N is derived entirely from tryptophan ring-N.

A 12- to 20-fold purification of tryptophan desmolase from *Neurospora* extracts was reported by Yanofsky (199). This enzyme, which is pyridoxol phosphate linked, catalyzes the reaction
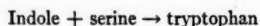
$$\text{Indole} + \text{serine} \rightarrow \text{tryptophan} \qquad 27.$$

A number of properties of the enzyme were described.

Isonicotinic acid hydrazide was found to inhibit indole formation from tryptophan by the tryptophanase of washed *E. coli* cells (200). The authors

suggest that isonicotinic acid hydrazide is acting as a competitive inhibitor of pyridoxal phosphate, the coenzyme of tryptophanase. However, this seems to be only a partial explanation because their data on coenzyme-inhibitor ratios suggest that there is also a noncompetitive inhibition.

Indole formation from tryptophan was reported for one *Pseudomonas* strain (201), but bacteriological examination of the culture later revealed it to be a typical *E. coli* (202). Thus all the pseudomonads so far studied decompose tryptophan by a peroxidative attack on the indole nucleus leading to kynurenine which is then decomposed to kynurenic acid by a route known as the "quinoline pathway," or more frequently by the "aromatic pathway," to anthranilic acid and β-keto adipic acid (190, 191).

Cystine, cysteine, and cysteine sulfinic acid.—An enzyme present in extracts prepared from washed bakers' yeast catalyzes a reduction of cystine to cysteine (203) by DPNH. TPNH[2] is inactive although the preparation also contains a TPNH-linked glutathione reductase. Cystine reductase could also be demonstrated in pea acetone powder and in *Candida albicans*.

A new coenzyme, coenzyme III, has been discovered as a result of studies on the metabolism of cysteine sulfinic acid in cell-free extracts of *P. vulgaris* (204, 205). It is required for the oxidation of cysteine sulfinic acid to the sulfonic acid, otherwise known as cysteic acid. The coenzyme has been purified by chromatography of boiled yeast juice and may be a nicotinamide mononucleotide pyrophosphate.

The sulfur products formed by a variety of soil microorganisms grown on cystine as sole carbon and sulfur source have been investigated (206). Pseudomonads produced an alkaline medium which contained tri- and tetra-thionates and thiosulfate. Fungi and various gram-negative, nonspore formers produced acid, and the final sulfur-containing products were sulfate and, in the case of fungi, dithionate.

Amino acid oxidation.—Further attempts to elucidate the mechanism of oxidative deamination of L-amino acids by *Clostridium sporogenes* and *C. saccharobutyricum* have been made using dialyzed cell-free extracts (207). DPN and phosphate are required for the oxidation. Arsenate will substitute for phosphate; this appears to be a true phosphate requirement and not a Na^+ or K^+ effect (Nisman).[4] TPN will not substitute for DPN.

The reduction of DPN by alanine can be followed spectrophotometrically and requires phosphate or arsenate. The reaction is reversed by the addition of pyruvate and ammonia. This suggests the existence of an alanine dehydrogenase acting in a manner similar to glutamic dehydrogenase except for the unexplained phosphate requirement. Whether or not this requirement is involved in the primary reaction remains to be investigated.

Creatinine degradation.—Studies on the bacterial decomposition of creatinine were described in a series of papers by Akamatsu *et al.* (208, 209, 210). Gram positive soil bacteria isolated from creatinine enrichment cultures produced an adaptive enzyme called creatinomutase that catalyzes the interconversion of creatinine and creatine. Cells heated at 55°C. for 30 min. to

destroy the creatinine decomposing system were used to demonstrate this interconversion. An equilibrium mixture contains approximately equal amounts of the hydrated open chain compound and the dehydrated ring form. The heated cell suspension also catalyzes the hydrolysis of glycocyamidine to glycocyamine; at pH 7.0 the reaction is almost completely in favor of the open chain compound. The enzyme catalyzed reactions are considerably faster than the corresponding nonenzymatic reactions.

Cell suspensions that have not been heated further decompose creatine to urea and sarcosine. The latter compound is metabolized to glycine, and finally, ammonia is liberated. Additional evidence against a preliminary demethylation of creatine to glycocyamine is derived from the observation that the latter compound is not decomposed in this system.

Peptides.—Upon closer inspection of their nutritional requirements, an ever increasing number of microorganisms have been found to require, under certain special conditions, one or more peptides for growth. Rational explanations for such requirements in a few cases have been reported recently (211 to 214).

S. faecalis was found by Snell and co-workers to require tyrosine peptides for growth in media containing high concentrations of pyridoxal (211). Under these conditions the tyrosine decarboxylase is very active and free tyrosine does not support growth. In media low in pyridoxal, where the tyrosine decarboxylase activity is very low, the organism grows equally well on tyrosine or tyrosine peptides. The implication to be drawn from these studies is that the peptides supply a constant source of tyrosine for protein synthesis whereas free tyrosine is rapidly decarboxylated in high pyridoxal media. The peptides are not decarboxylated.

Hirsch & Cohen (212) found that valine-requiring *E. coli* mutants use valyl peptides equally well for growth and are insensitive to a variety of amino acids that act as competitive inhibitors of free valine when valine is furnished as a peptide.

A similar effect was noted by Marshall & Woods (213) using a variety of tryptophan requiring organisms. Although the organisms used tryptophan or some tryptophan dipeptides equally well for growth, the dipeptides were more efficient in overcoming the inhibition caused by 4-methyl tryptophan.

L. casei requires pyridoxal for growth but if D-alanine is added to the medium there is no longer a pyridoxal requirement. Apparently the addition of pyridoxal is necessary only for the formation of D-alanine, presumably by a pyridoxal-phosphate catalyzed racemization of L-alanine, which is supplied in the medium. However, when the organism is grown on D-alanine (L-alanine present) there is now a requirement for L-alanine peptides (214). From these results it is concluded that D-alanine inhibits the utilization of free L-alanine but not of L-alanine peptides. Since the peptides are actively split to free alanine by cell suspensions, it is postulated that the inhibitory effect of D-alanine may be exerted at the cell surface preventing the entrance of free L-alanine but not of L-alanine peptides.

Steroid Oxidations

Recognition of the physiological importance of cortisone together with its widespread clinical use has greatly stimulated interest in microbial oxidative processes as a means of converting readily available steroids into compounds more closely related to cortisone. The feasibility of such an approach seemed apparent from a number of earlier studies showing that a wide variety of bacteria and fungi are able to carry out limited oxidations of steroids. This earlier work has been reviewed recently by Arnaudi (215).

An extensive series of investigations carried out by the research group of The Upjohn Company (216 to 223) have shown that various fungi of the Order Mucorales, especially certain *Rhizopus nigricans* and *R. arrhizus* strains, are admirably suited for the introduction of an oxygen into position-11 of a variety of steroids. For example, progesterone, a relatively available steroid, can be oxygenated to 11-α-hydroxyprogesterone in essentially quantitative yields by *R. nigricans* (217). Using this product as starting material, 17-α-hydroxy corticosterone acetate (Kendall's Compound F) can be prepared in an over-all yield of 15 per cent by a series of straightforward chemical reactions (224). Similarly, 17-α, 21-dihydroxy-4-pregnene-3,20-dione (Reichstein's Compound S) is converted in good yield to the 11-epimer of Compound F by *R. nigricans* (220). The 11-epimer compound is easily oxidized to cortisone acetate in good yields. A number of new oxygenated derivatives of several steroids have been isolated and identified as a result of these studies.

In addition to these limited oxygenations of steroids, a number of microorganisms are known to degrade these compounds much more extensively. This is evident from the fact that they can serve as sole carbon sources for growth (225 to 228). The utilization of compounds lacking a C_{17} side chain shows that the cyclopentenophenanthrene nucleus of steroids must undergo bacterial degradation. Recent studies employing pseudomonads (229, 230, 231) and a *Mycobacterium* (232) provide direct evidence that this is indeed the case. Chemical, radioactive, and manometric data all show that growing cultures and cell suspensions oxidize certain sterols completely to carbon dioxide. Soluble extracts, however, have not yet been demonstrated to form carbon dioxide from a sterol. This may be a result of failure to solubilize the terminal oxidase system.

The oxidation of steroid alcohols to the corresponding ketones (i.e., cholesterol to Δ^4-cholestenone or testosterone to Δ^4-androstene-3,17-dione) is catalyzed by cell-free extracts of various bacteria. The enzyme is distinct from ethanol dehydrogenase. Extracts of the pseudomonas studied by Talalay *et al.* (229), contain a DPN specific enzyme that oxidizes testosterone to Δ^4-androstene-3,17-dione. The reaction can be followed spectrophotometrically at 340 mμ.

The status of our knowledge concerning the mechanism of oxidative degradation of the cyclopentenophenanthrene nucleus of steroids is similar to that of fatty acid oxidation about 10 years ago. Aside from two acids that are the result of oxidative cleavage of ring A, one a keto acid isolated by

Turfitt (233) and another a dicarboxylic acid isolated by Stoudt & Brower (234), there is no information concerning intermediates occurring between the C_{19} steroids and CO_2. The mechanism whereby the side chain carbons of a compound such as cholesterol are converted to CO_2 is equally obscure. Since intermediates in these oxidative processes seem to accumulate in only trace amounts, if at all, it is probable that very little progress can be expected until active soluble extracts are obtained.

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BIOCHEMICAL ASPECTS OF VIRAL GROWTH[1,2]

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Introduction

A concept currently accepted generally is that viruses alter the metabolism of the host cells to favor the production of new virus particles to the detriment of the host cell. The various theories concerning the precise nature of these particles will not be discussed here.

The metabolism of viral replication must involve energy exchange. Much of the experimental work at present is directed towards attempts to identify the chemical systems involved in this exchange of energy and particularly those which are essential to the process. From the chemist's standpoint, it is difficult to solve the problem unless the viral and cellular components involved can be identified quantitatively.

Previous work related to isolation and purification of virus has been reviewed extensively during recent years by Beard (1, 2, 3), Knight (4, 5), Lauffer, Price & Petre (6), Pirie (7), and Anderson (8). It is apparent from these reviews that there are many difficulties involved in virus purification. The present deficiency in knowledge concerning normal cellular physiology needs no elaboration. Consequently, much of the work to date concerning metabolism of host-virus systems has been of an exploratory nature and often has been only qualitative in character. The system studied most extensively has been that of the bacteriophage-bacterial host cell. This work was summarized by Price (9) last year and will not be considered here. It should be emphasized that most of the studies on bacteriophage have been concentrated on a single group, the coliphages. The findings of these studies may not apply to other phage systems and will not necessarily have any analogies with other viruses.

Much attention has been focused on special properties of viruses which basically must depend on chemical specificities. The interference phenomenon is an example. This work has been discussed recently for animal viruses by Lennette (10) and for plant viruses by Bennett (11). Hemagglutination and hemagglutination-inhibition in relation to possible enzymatic activities

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[1] The survey of literature pertaining to this review was terminated in January, 1953.

[2] The following abbreviations are used in this chapter: PABA (*para*-aminobenzoic acid); PGA (pteroylglutamic acid); CF (citrovorum factor); DNA (desoxyribonucleic acid); RNA (ribonucleic acid); MP (meningopneumonitis); TMV (tobacco mosaic virus); BAL (2,3-dimercaptopropanol).

of the virus concerned also have been reviewed recently by Burnet (12). Studies concerning the effects of various chemicals on virus-host cell systems were reviewed by Eaton (13) in 1950.

The present paper is concerned primarily with metabolic inhibitors of virus propagation. Various chemicals have been tested *in vitro* or *in vivo*, with or without studies of possible mechanisms involved. In some instances it is not known whether the chemical is exerting its action primarily on the virus, on the host tissue, on tissue metabolites, or possibly on a product of host-virus interaction. Often theories to explain the reaction observed are suggested but no substantiating studies are made to establish the validity of the assumption. The problem of antimetabolites is complex under any circumstances; the general principles involved have been analyzed by Shive (14) and by various authors (15). There are many difficulties in evaluating experiments of this type in chemically undefined systems of virus-host cells. The studies considered here are arranged according to the virus or group of biologically related viruses used. This was done in preference to discussion of the problem on the basis of the biochemical systems involved, since at present the data comprise observations of a group of heterogeneous agents without clearly defined metabolic relationships.

PSITTACOSIS GROUP

Sulfonamides are effective *in vitro* and *in vivo* against some members of this group. However, there is considerable variation among the various organisms in regard to sensitivity to a given agent. The mechanism of drug inhibition on the growth of psittacosis virus in the yolk sac of chick embryos has been studied by Morgan (16). He found that para-aminobenzoic and pteroic acids antagonized sulfadiazine inhibition of growth in a competitive manner, that is, there was a direct relationship between the inhibiting dose of drug and the infecting dose of virus. Pteroylglutamic acid exerts a non-competitive antagonism. Some analogues of PGA[2] also antagonized sulfonamide inhibition of virus but neither PGA nor PABA[2] antagonized penicillin inhibition. Reasoning by analogy with the theories of action on bacteria, the author suggests that sulfonamide interferes with the use of PABA in the synthesis of PGA by the virus, which requires the latter compound for growth. Pteroic acid would possibly be an intermediate in PGA synthesis. The citrovorum factor but not Vitamin B_{12} also antagonized sodium sulfadiazine (17). In embryonated eggs certain analogues of PGA such as 9-methylpteroylglutamic acid and 4-aminopteroylaspartic acid inhibited growth of psittacosis virus. The latter compound among other analogues was shown by use of the tissue culture technic not to exert this action by reason of direct tissue toxicity. The PGA analogue inhibition of virus was reversed by the addition of CF.[2] Since psittacosis virus contains a considerable amount of desoxyribonucleic acid (DNA) and because of the possible role played by PGA and CF in DNA[2] synthesis by bacteria, it is suggested that similar processes may be involved in this viral synthesis. Meningopneumonitis virus

propagation in embryonated eggs and tissue culture also was inhibited by 4-aminopteroylaspartic acid.

Golub (18) also found that PABA antagonized sulfadiazine inhibition of the psittacosis virus but not of lymphogranuloma venereum virus. The effect of various enzyme inhibitors on psittacosis virus propagation was studied by Burney & Golub (19). Several compounds including hydroquinone, proflavine, and quinacrine hydrochloride (atabrine) inactivated virus by direct contact *in vitro* whereas various other substances including azide, cyanide, and urethane did not have this effect. Chick embryo tissue exposed to o-iodosobenzoate did grow in tissue culture subsequently but did not support growth of virus. After contact with hydroquinone the tissue would also grow in tissue culture but supported only poorly the propagation of virus. The suggestion was made by these authors that viruses susceptible to a given drug may contain similar enzyme systems.

The effects of certain compounds on psittacosis propagation in eggs were studied by Morgan (20). Various purines and pyrimidines did not influence sulfadiazine inhibition. Purine analogues including benzimidazole, 2,6-diaminopurine and 8-azaguanine inhibited viral growth in cultures of chick embryonic tissue. Adenine reversed the diaminopurine inhibition and guanine reversed the azaguanine inhibition. The pteridines, 2-amino-4-hydroxy-6-formylpteridine, and xanthopterin inhibited virus while some related compounds did not. The inhibition by the first named was reversed by xanthine, although there was no correlation found between inhibition and xanthine oxidase activity. The possibility that the pteridines might act by interference with utilization of PGA was suggested but not studied. These studies were extended to include some pyrimidines (21). Although uracil, 6-methylthiouracil, 6-methyluracil and diazouracil had no effect on psittacosis virus growth in tissue culture, thiouracil did inhibit viral growth. The possibility was suggested by the author that ribonucleic acid synthesis is involved in the growth of psittacosis virus. To test this hypothesis, it would be desirable to know whether or not uracil reverses the inhibition by thoiuracil and what influence these reactions have on the RNA[2] content of infected and non-infected cells. The mechanism by which lipid fractions of normal sera inactivate psittacosis virus *in vitro* as reported by Utz (22) remains to be explained.

Eaton, van Allan & Wiener (23) found that acroflavine and two acridine compounds inhibited mouse and cat pneumonitis, lymphogranuloma venereum, and meningopneumonitis viruses in embryonated eggs. Proflavine and quinacrine hydrochloride were not inhibitory. Various substituted nitrobenzenes including chloramphenicol also inhibited these viruses according to Eaton, Huang & Levenson (24). Chloramphenicol, aureomycin, and terramycin were effective against feline pneumonitis virus in mice [Kneeland & Price (25)]. Wells & Finland (26) reported that aureomycin and chloromycetin prolonged the life of chick embryos infected with psittacosis virus.

The inhibition of MP[2] virus in embryonated eggs by a nitrofuran was

reversed by cysteine [Oddo & Eaton (27)]. Cysteine did not reverse the inhibition of chloramphenicol or aureomycin. These findings suggest that —SH groups are concerned in the inhibition but what particular enzymes may be involved is not known.

Dickinson & Inkley (28) tested the effect of 250 compounds on sheep abortion virus in embryonated eggs. Only terramycin and chloromycetin were inhibitory.

VACCINIA

The capacity of various agents to inhibit the propagation of vaccinia virus in cultures of embryonic chick tissue was studied by Thompson (29). Succinic acid and various substances involved in intermediate carbohydrate metabolism had no effect on the virus. Pantothenic acid and nicotinic acid analogues as well as desthiobiotin were without effect, but ascorbic acid decreased the amount of virus. PABA did not alter viral growth but several amino acid analogues including those of glycine, methionine, phenylalanine and valine were inhibitory. Compounds of particular tissue toxicity, such as cyanide, azide, dinitrophenol, and iodoacetic acid were inhibitory for virus as were also several quinones, quinacrine hydrochloride, and proflavine. To what extent any of the above mentioned compounds were toxic for the host tissue is not stated. These studies were extended to include various purine analogues by Thompson and his associates (30). Benzimidazole and certain halogenated purines were inhibitory for virus and this action was not antagonized by the presence of adenine. However, the inhibition by 2,6-diaminopurine was reversed by adenine at an equivalent concentration and also by adenylic, guanylic, and yeast nucleic acids and by hypoxanthine and diphosphopyridine nucleotide at higher concentrations. Guanine, xanthine, or uracil were inactive as reversing agents. 8-Azaguanine and some related compounds failed to inhibit virus. Although compounds such as 2,6-diaminopurine and benzimidazole were active *in vitro*, they were inactive against vaccinia infection in mice. The latter findings were in contrast to other observations of Thompson *et al.* (31) that some 5-phenoxythiouracils were only moderately active viral inhibitors *in vitro* but reduced the mortality in mice treated with these compounds.

Hamre, Bernstein & Donovick (32) found that *para*-aminobenzaldehyde, 3-thiosemicarbazone interfered with vaccinia infection of chick embryos and mice although it was not active against agents of meningopneumonitis or swine influenza virus. Benzaldehyde, 3-thiosemicarbazone and the *para*-amido, -amino, -methoxy, -propoxy, and -ethylsulfonyl analogues were also effective against vaccinia in chick embryos according to the report of Hamre, Brownlee & Donovick (33). In chick embryo tissue cultures Thompson, Price & Minton (34) found that substitutions in the *para* position of the benzene nucleus or in the 4- position of the thiosemicarbazone portion of the molecule reduced virostatic activity. Benzaldehyde, 3-thiosemicarbazone protected mice inoculated with vaccinia virus.

Gohar & Bashatli (35) found that penicillin inhibited vaccinia in rabbits.

Lee (36) did not find any inhibition of vaccinia or myxoma viruses in chick embryos treated with aureomycin, chloramphenicol, or *para*-aminobenzoic acid. Compounds structurally related to chloramphenicol, such as some of the alpha-haloacylamides were reported by Thompson and associates (37) to inhibit vaccinia virus, *in vitro*.

The mechanisms of inhibition involved in the compounds found effective in the above studies are unknown. It would be premature to assume that competitive metabolites are involved in the case of the purine analogues in the absence of additional information such as quantitative studies of the ratios of concentration of analogue to metabolite.

HERPES

Ackermann & Francis (38) studied herpes infection in chick embryos. They found that viral infection stimulated proliferation of the liver and heart host cells associated with increased nucleic acid synthesis by the cells. In chick liver there was a change in the normal ratio of succinoxidase to α-ketoglutaric acid oxidase activity. Both of these enzymes presumably are associated with mitrochondrial particles. Herpetic infection of the heart was accompanied by a decrease in the activities of the above mentioned enzymes. In an extension of these studies Ackermann & Kurtz (39) found that 80 per cent of the virus in the cytoplasm of infected cells was not combined with mitochondria or nuclei; 16 per cent was closely attached to mitochondria. Since there is mitochrondrial degeneration in virus infected cells, it was suggested that these cellular units are associated with virus proliferation. Francis & Kurtz (40) did not find evidence to indicate that herpes virus was selectively bound to the nuclear protein of infected cells. The results indicated that the cellular intranuclear inclusions associated with herpetic infection are not herpes virus. A different conclusion was reached by Crouse *et al.* (41) on the basis of histochemical stains of infected chick membranes. They described an early accumulation of desoxyribose nucleoprotein in inclusion bodies. Older inclusion bodies did not have the nucleoprotein. The presence of desoxyribose nucleoprotein in the inclusion was interpreted as evidence that the latter is an aggregation of virus material.

Blank, Kaneda & Liu (42) noted an increased amount of P^{32} in the amniotic fluid of eggs infected with herpes or vaccinia. P^{32} was concentrated in the areas of reaction on the membrane. Such areas could result either from viral infection or mechanical irritation and should not be considered to be the result of a specific viral activity.

Antibiotics such as aureomycin were not effective against herpes virus in embryonated eggs in studies of Baldridge & Blank (43).

INFLUENZA

Chemical changes in relation to infection with influenza virus have been studied by Kilbourne & Horsfall (44). They found that an increase in protein content of the allantoic fluid occurred during the first two days of infec-

tion. This increase roughly parallels the rise in hemagglutination titer. Kalter (45) did not find an increase in protein but did note differences in the concentrations of amino acids in normal egg allantoic fluid and in fluid from eggs infected with the PR8 or Lee strains of this virus. He suggested that a particular virus altered the protein synthesis of the host tissues which would be reflected in a rearrangement of the amino acids according to the needs of the virus. Eaton et al. (46) observed that certain amino acids such as arginine, lysine and ornithine inhibit the growth of influenza virus in tissue cultures of chorio-allantoic membrane. This inhibition was not attributed to direct action of the chemicals either on the host tissue or the virus, and occurred in the absence of changes in oxygen consumption, glucose utilization, and growth potential of the tissue. The inhibitory effect of arginine could be reversed with egg yolk. The inhibitory effect of lysine polypeptides on the growth of influenza virus noted by Rubini, Stahmann & Rasmussen (47) was perhaps related to mechanical interference with the virus-host cell since these substances cause aggregation of virus particles. Ackermann (48) found that methoxinine and ethionine inhibit propagation of the PR8 strain in tissue cultures of chick embryo. Since the action did not seem to be directed against the host tissue or the virus and since the inhibition was reversed by methionine it was suggested that the latter is involved in the biosynthesis of virus. Homocysteine partially reversed inhibition by methoxinine and ethionine but betaine was inactive. Several α-aminosulfonic acids were found by Ackermann (49) to inhibit virus propagation in tissue culture as well as in embryonated eggs. These sulfonic acids were not viricidal nor did they affect the tissue repiration. The inhibition was not affected by structurally related substances, amino acids such as tyrosine or phenylalanine. It was postulated that these inhibitors conceivably might act through blocking enzymes of limited substrate specificity.

Ackermann (50) failed to find any differences in respiration of chorio-allantoic membranes cultured *in vitro* in the presence or absence of the PR8 strain. Virus production was inhibited by reduced oxygen tension. Malonic acid and antimycin inhibited endogenous tissue respiration and viral propagation. It was suggested that this action could be through inhibition of succinic dehydrogenase. Further evidence suggesting that a mechanism of inhibition was related to the citric acid cycle was the finding that sublethal doses of fluoroacetate tended to block the citric acid cycle in the mouse lung (51). This was associated with inhibition of virus propagation in the lungs of treated mice. Fluoroacetate itself was not active *in vitro* against virus. The dependence of virus propagation on respiration of the host tissue was likewise shown by the work of Daniels, Eaton & Perry (52). Deprivation of glucose markedly reduced the production of influenza virus in chick embryo membranes. Pyruvate could only partially substitute for glucose in stimulating virus propagation.

Phosphorus metabolism was studied in chick embryos infected with influenza virus by Parodi (53). He found that infection was associated with a

reduction in phosphocreatine. Graham & McClelland (54, 55) obtained fluid from eggs infected with the PR8 strain of influenza virus in the presence of P^{32}. The virus was partially purified. Most of the P^{32} was found in the pentosenucleic acid fraction. The evidence suggested that isotope was contained in the virus; however, there was not sufficient material obtained to determine the specific activity. Approximately 94 per cent of the phosphorus recovered was in the pentosenucleic acid fraction. Large amounts of P^{32} were found to be deleterious to the chick embryo but did not interfere with virus production. It was calculated that the specific activity would have to be increased by at least one thousandfold in order to obtain labelled virus suitable for tracer work. Tracer studies of de-embryonated egg membranes with the PR8 strain have also been reported by Cohn (56). He found that virus infection is associated with accelerated incorporation of P^{32} into the membrane, a decrease in the phospholipid phosphorus of the membrane and an increase of lipid-bound phosphorus in the fluid medium.

A variety of viral antagonists have been studied by various workers. Cushing & Morgan (57) found that analogues of pyridoxine and thiamine, desoxypyridoxine and oxythiamine inhibit the PR8 strain in cultures of chick embryo. Analogues of nicotinic acid, pantothenic acid, phenylalanine, xanthine, adenine and guanine were noninhibitory. Since the antagonists were not added to the culture until after 24 hours the effects would seem to result from intracellular activity rather than from interference with access of virus to the cells. Fastier (58) reported that infection in chick embryos was not influenced by hyaluronidase. Most of the antibiotics tested have not altered influenza virus infection. However, under certain circumstances terramycin may alter infection in chick embryos [Kass, Lundgren & Finland (59); Vinson & Walsh (60)].

Various compounds including pyridines, pyrimidines, amino acids, quinones and quinilines were not effective against PR8 infection in mice [Wooley, Bond & Perrine (61)]. However, Rasmussen & Stokes (62) and Eaton, Cheever & Levenson (63) found that certain acridines inhibited the PR8 and Lee strains of influenza virus in tissue culture and in embryonated eggs but not in mice. Briody & Stannard (64) found that proflavine inhibited influenza B but not A virus in eggs. Certain aromatic diamidines inhibit influenza virus in eggs and chick embryo tissue cultures as shown by McClelland & Van Rooyen (65), Eaton et al. (66), and Eaton, Levenson & Perry (67).

Hoyle (68) found that some triphenylmethane dyes inhibited influenza virus in chick embryos. He suggested that possibly the dye interferes with RNA metabolism of the infected cell since dyes are known to combine with ribonucleic acid, a component of the virus [Knight (69)]. Fleisher (70) found that certain quinone-imide dyes inactivated virus *in vitro* but had little effect in altering infection in mice unless they were used in concentrations near the lethal dose. Schmidt & Rasmussen (71) found that inhibition of influenza virus in eggs by cobaltous ions could be reversed by histidine, cysteine or

sodium thioglycolate. This suggested interference with —SH or imidazole groups. Pérez, Baralt-Pérez & Klein (72) found that mercuric chloride inhibition of the PR8 strain in chick embryos could be reversed by BAL.[2] The mechanism of this action was not determined. Crude extracts of tea were found by Green (73) to inhibit the PR8 strain in eggs. The specific component or components involved were not determined. Fong & Bernal (74, 75) reported that certain mustards inactivated the infectivity and toxicity without much alteration of the antigenicity of influenza virus. Groupé, Pugh & Levine (76) describe a substance derived from *Achromobacter sp* which was not active, *in vitro*, against influenza virus but which suppressed pulmonary lesions in mice infected with influenza or with Newcastle disease viruses. The nature of the substance is under investigation.

MUMPS

Gardner & Morgan (77) found differences in the activity of certain enzyme inhibitors for the hemolytic and hemagglutinating activities of mumps virus. Thus, hemolysin but not hemagglutinin was inhibited by hydroxylamine hydrochloride or urethane. Cysteine increased both activities of the virus while glutathione potentiated hemagglutinin and inhibited hemolysin. The possibility that hemolysin is a lecithinase was discussed. The principal known difference at present is the heat stability of lecithinase A in contrast to the relative heat lability of mumps hemolysin. Cushing & Morgan (57) state that oxythiamine or desoxypryidoxine inhibited mumps as well as influenza virus in tissue cultures of chick embryo. Agents such as 2,6-diaminopurine and 8-azaguanine were not inhibitory. Briody & Stannard (64) reported that proflavine in concentrations sufficient to inhibit vaccinia or type B influenza virus in eggs did not inhibit mumps virus. Certain aromatic diamidines inhibit mumps as well as other viral agents in tissue cultures and in chick embryos according to Eaton *et al.* (66, 67).

POLIOMYELITIS

The factors affecting the growth of poliomyelitis virus in tissue culture have been reported in detail by Weller *et al.* (78) and by Robbins, Weller & Enders (79). The use of protein-free nutrient media for this purpose was discussed by Melnick & Riordan (80). Such a medium was suitable for propagation of monkey testicular tissue since it supported a good growth of fibroblasts. Another type of synthetic medium was also found useful by Wood *et al.* (81) for use with testicular tissue. By use of this medium Franklin *et al.* (82) found that tissue infected with Lansing virus used less glucose than control tissue. Since viral infection causes tissue necrosis this is possibly a phenomenon associated with tissue degeneration. Brown & Ackermann (83) reported that ethionine inhibited Lansing virus in cultures of human embryonic brain tissue. Methionine partially reversed this inhibition. Betaine or homocysteine did not reverse the action of ethionine. It was concluded that methionine was essential for Lansing propagation in this system.

Ainslie (84) found that a methionine antagonist, methionine sulfoximine, inhibited the growth of Lansing virus in mice and prolonged the incubation period. It would be of interest to know if the inhibitory action of the sulfoximine could be reversed by methionine. Sodium monofluoroacetate likewise produced an inhibition of virus.

In experiments described by Gershoff et al. (85) excess dietary methionine decreased the susceptibility of mice to Lansing virus. The incubation period and survival time of such mice were increased. The protective effect was enhanced by addition of 6-methyl tryptophan to the diet. The latter substance given by itself offered some protection against infection. Studies were made by Davies et al. (86) of the effects of deficiencies of the essential amino acids on susceptibility of mice to infection with Lansing virus. It was concluded that deficiencies of methionine, tryptophan, isoleucine, and valine suppressed infection.

Brown (87) reported that various amino acid analogues as well as proflavine, benzimidazole, and 2,6-diaminopurine inhibited Lansing virus in cultures of monkey testicular tissue. The inhibitory effect of thienylalanine was partially reversed by phenylalanine. The inhibition by benzimidazole was not reversed by adenine or guanine alone but was partially reversed by a mixture of adenine, guanine, and uracil. Adenine or guanine alone partially reversed inhibition by 2,6-diaminopurine. Homobiotin was the only vitamin analogue found to be inhibitory.

Little & Lawson (88) reported that Lansing virus infection of mice resulted in decreased cholinesterase activity of spinal cord homogenates in comparison with noninfected tissue. It was felt, however, that these changes could be explained by the increased nitrogen content of the infected tissue.

Cortisone but not ACTH, progesterone, or diethylstilbestrol altered infection by the MEF_1 or Lansing strains in hamsters. The incubation period was shortened and the disease produced was more severe in treated animals. Schwartzman & Fisher (89) pointed out that cortisone has been reported to alter susceptibility to other virus infections but the mechanisms involved are not necessarily the same in each instance.

ENCEPHALOMYELITIS

Siem, Smith & McLimans (90) reported that embryonated eggs infected with Western equine encephalomyelitis virus had a marked drop in oxygen uptake beginning approximately 20 hours after infection. Since all infected eggs were dead by 36 hours this alteration in oxygen uptake probably was related to the terminal stages of cell necrosis in the embryo. Friend (91) found that 2,6-diaminopurine inhibited the growth of Russian encephalitis virus in tissue cultures of mouse tumor. The inhibition could be reversed by adenine but not by guanine. Moore & Friend (92) found that 2,6-diaminopurine increased the survival rate of mice infected with Russian encephalitis virus.

Watanabe, Higginbotham & Gebhardt (93) reported that sodium mono-

fluoroacetate prolonged the survival time of mice infected with Eastern equine encephalomyelitis virus. The drug did not have an *in vitro* effect on the infectivity of the virus nor an *in vivo* effect on susceptibility of the host. It was suggested that the drug possibly interferes with virus replication by inhibition of the citrate cycle since the latter is known to occur. None of the various chemicals studied by Wooley, Bond & Perine (61) influenced St. Louis encephalitis virus infection in mice.

MURINE ENCEPHALOMYELITIS

Factors affecting the propagation of Theiler's GD VII strain of mouse encephalomyelitis virus in flasks of minced, one-day-old mouse brain were studied by Pearson (94). Certain heavy metals as well as enzyme inhibitors such as cyanide, azide, iodoacetate, and dinitrophenol inhibited growth of virus. Pearson & Winzler (95) reported that propagation of the virus did not alter the oxidative or glycolytic metabolism of the host tissue. However, Rafelson, Winzler & Pearson (96) noted that virus infection markedly stimulated the turnover of total protein-bound phosphorus. This turnover was primarily concerned with RNA phosphorus rather than DNA phosphorus. The virus effect on phosphorus was different for brains of mice of different ages (97). Virus infection likewise stimulated the incorporation of glucose fragments into the protein fraction of tissue and decreased incorporation into the lipid fraction (98). In studies of amino acid metabolism (99) the presence of virus stimulated incorporation of radioactive carbon from glucose into most of the amino acids except proline and threonine but inhibited the incorporation of glucose fragments into lysine and histidine. The latter two amino acids were present in smaller amounts in virus infected tissue than in control tissue. These results suggested that virus propagation might be associated with lysine and histidine metabolism. Other data presented by Winzler *et al.* (100) indicated that degradation products of amino acids in one-day-old mouse brain may be in metabolic equilibrium with intermediates derived from glucose. This could result in the appearance of radiocarbon from glucose in essential amino acids with no net synthesis of the amino acids. Probably free amino acids were necessary intermediates between radioglucose and labelled protein-bound amino acids. Pearson, Lagerborg & Winzler (101) found that various amino acids and analogues inhibited Theiler's virus in minced, one-day-old mouse brain. Lysine was the most active amino acid. Partial reversal of lysine inhibition was obtained with methionine, leucine or tyrosine. Lysine, and to a lesser extent, histidine in addition to inhibiting virus propagation also inhibited the incorporation of radiophosphate into phospholipid and protein-bound fractions (102). This inhibition of phosphate conversion also was partially reversed by the presence of methionine. Thienylalanine inhibited virus growth but had little effect on radiophosphate turnover. Visser, Lagerborg & Pearson (103) reported that various nucleoprotein derivatives including adenine, cytidine, and thymine also inhibited the propagation of Theiler's virus *in vitro*. Guanine, uridine, or uracil were not

inhibitory. Various substituted uridines were inhibitory. This effect was partially reversed by uridine. 2,6-Diaminopurine was not inhibitory. Benzimidazole inhibited virus propagation and radiophosphate uptake whereas 8-azaguanine did not inhibit either growth or uptake (102). The action of the substituted nucleoside 5-chlorouridine was further studied by Rafelson, Pearson & Winzler (104). In addition to inhibiting the virus this compound inhibited the uptake of radiophosphorus by the RNA fraction of minced one-day mouse brain. Both these inhibitory effects were partially reversed by the action of uridine. Since virus propagation was associated with both an increased turnover and increased net amount of RNA phosphorus, it was suggested that RNA was intimately concerned with the metabolism involved in the propagation of Theiler's virus in mouse brain.

In vivo effects of various amino acid deficiencies on resistance to infection with Theiler's GD VII virus were studied by Kearney *et al.* (105). Tryptophan deficiency resulted in a modified disease with suppression of signs of infection. This was not due to inhibition of virus multiplication in the brain. Deficiencies of certain other amino acids tested by Pond *et al.* (106) likewise reduced the incidence of paralysis or prolonged the incubation period without reducing the eventual fatality rate. The rate of multiplication of virus in the presence of tryptophan-deficient mice was slower than in nondeficient animals.

Little & Lawson (88) found that the cholinesterase activity of brain homogenates of mice was inhibited in the presence of Theiler's FA encephalitis virus. Jungeblut (107) reported that certain naphthoquinonimines were the most effective of many compounds tested in preventing death of mice infected with Columbia₁-SK virus.

TOBACCO MOSAIC VIRUS

Considerable work has been done on purification and analyses of plant viruses. There has been much discussion of the mechanisms of virus production [Lugg (108); Pirie (109)]. Only recently has much experimental work been directed toward an experimental study of the problem. Various compounds have been found to inhibit the multiplication of plant viruses. Matthews (110) inhibited the growth of Lucerne mosaic virus in tobacco plants by spraying guanazolo on the leaves. This inhibition could be reversed by adenine, guanine, or hypoxanthine but not by xanthine, uracil, caffeine, or related compounds. Triazolo showed some inhibition of virus in the tobacco plant but was more active than guanazolo in the bean plant. However, guanazolo was toxic for the bean plant. The possible role of adenine, guanine, and hypoxanthine in the synthesis of virus was discussed. Naphthaleneacetic acid but not folic acid inhibited production of virus in tissue cultures of tobacco plants according to Kutsky & Rawlins (111). Kutsky (112), in an extension of this work found that only indolebutyric acid of several compounds tested inhibited tobacco mosaic virus in tissue cultures. Both naphthaleneacetic acid and indolebutyric acid can function

as synthetic growth hormones, and growth stimulation of the host tissue cultures was noted. Takahashi (113) reported that malachite green inhibited TMV² propagation in detached tobacco leaves. This effect was considered to be achieved by inhibition of undetermined enzymes. Stahmann *et al.* (114, 115) found that synthetic lysine polypeptides reversibly inhibited TMV. This action resulted from combination of the peptides with and precipitation of TMV. Commoner & Mercer (116, 117) reported that TMV synthesis in isolated tobacco leaf tissues was inhibited by thiouracil. This inhibition was partially reversed by uracil. It was pointed out that these data do not necessarily indicate interference with nucleic acid metabolism since thiouracil may inhibit other systems such as tyrosinase activity. Commoner (118), in studying nitrogen metabolism associated with TMV production, reported that synthesis of virus induces withdrawal of host nonprotein nitrogen. Several specific, soluble low molecular weight proteins are synthesized along with virus in infected tissue. Virus protein nitrogen is derived from free ammonium nitrogen without passing through a pool of free amino acids and amides, consequently virus protein synthesis involves *de novo* formation of peptide bonds either from ammonia or a component derived from ammonia which is not free amino acid or amide nitrogen. The nonvirus soluble proteins synthesized *de novo* in infected tissue may represent virus precursors. The site of synthesis of virus of or its protein precursors is probably an insoluble cell particulate. Wildman, Cheo & Bonner (119) presented data which suggested that TMV protein was synthesized at the expense of normal nucleoprotein, since there was a simultaneous and proportional decrease in normal nucleoproteins as the virus protein was formed. Meneghini & Delwiche (120) cited evidence that TMV protein once formed is not in equilibrium with host cell constituents. Studies of the process of mutation during TMV production were described by Knight (121). This would seem to occur through changes in the composition of protein rather than through changes in nucleic acids. This idea is in accord with previous data that mutation is associated with alteration in the proportion or kind of amino acids in the virus protein. Present studies revealed little variation in the purines and pyrimidines.

MISCELLANEOUS GROUP

Ward (122) found that infection of the chick embryo with the common cold virus resulted in an alteration in phosphorus metabolism. Infection resulted in an increased rate of incorporation of radiophosphate into RNA. Similar changes were found in eggs infected with influenza and mumps viruses. Smith, Murphy & Mirick (123) reported that ACTH increased virus growth of pneumonia virus in mice without alteration of the infection. Cortisone, however, enhanced both virus multiplication and infection. Groupé *et al.* (124) reported that viscosin, an antibiotic from *Pseudomonas sp.*, inhibited infectious bronchitis virus infection in embryonated eggs and had slight suppressive effect on mice infected with influenza virus. Eaton, Perry & Gocke (125) showed that chloramphenicol inhibited atypical pneumonia

virus in chick embryos but not in cotton rats; certain other nitro compounds had a variably inhibitory effect on virus in chick embryos but a markedly protective effect in cotton rats.

Bauer (126) studied the nucleic acid metabolism of brains of mice infected intracerebrally with various viruses. These included yellow fever, lymphocytic choriomeningitis, vaccinia, and dengue. Of the enzyme systems studied, the activity of certain enzymes was increased by virus infection and possibly was characteristic for a particular virus. In most systems increased activity was associated with the pathway hypoxanthine to adenylic and guanylic acids. Fox (127) studied factors affecting growth of yellow fever virus in culture of chick embryo tissue. Diminished oxygen tension inhibited virus production. Mommaerts *et al.* (128) reported that partially purified preparations of avian leucosis virus were active in dephosphorylating ATP. This suggested a possible mechanism whereby energy-rich compounds might be available for virus growth.

Myxoma virus infection in chick embryos is associated with increased glycolysis of the chorioallantoic membrane, possibly by activation of a glycolytic enzyme (zymohexase) according to the work of Kun & Smith (129).

Chinn (130) found that certain antibiotics, including terramycin and aureomycin, could inactivate Rous sarcoma virus *in vitro*. Andrewes & Niven (131) reported aureomycin and terramycin effective against the grey lung virus infection of mice. Penicillin, streptomycin, and chloromycetin were not effective.

Infection of embryonated eggs with the viruses of lumpy skin disease or of blue tongue resulted in increased protein content of the egg fluids [Polson & Dent (132)]. Electrophoretic patterns established the appearance of definite peaks associated with virus.

McLimans *et al.* (133) observed a relationship between the rate of oxygen uptake by embryonated eggs and the rate of proliferation of Newcastle disease virus in the eggs. Viral growth did not stimulate oxygen uptake.

Bird (134) has described the formation of polyhedral bodies in the cells of infected insects in relation to the rods and spheres thought to be the viral particles. The polyhedra develop within masses of coagulated nuclear chromatin and are known from previous work to consist mostly of noninfectious material. Wellington (135) has shown that the amino acids obtained from proteins of the polyhedra of silkworms or from the capsule associated with a virus infecting *Cacoecia murinana* (Hb.) are qualitatively similar to the amino acids recovered from the partially purified viruses. Quantitative studies are necessary to determine if differences may exist.

Discussion

Even a cursory reading of the papers cited here should convince the reader that most workers are well aware of the desirability of obtaining more precise information concerning systems under study. This will be a laborious task under any circumstances and it is particularly difficult at present be-

cause sufficient data often are not at hand to indicate which biochemical processes should be selected for detailed investigation. The current trends of study in the field are fairly obvious. Most workers have in mind the findings that nucleic acid and protein are the only constituents common to all viruses thus far analyzed [Knight (4)]. Consequently, studies of intermediary metabolism of protein and of nucleic acids of host tissue should yield useful information concerning processes associated with viral growth.

Perhaps more attention has been given to amino acid metabolism than is justified simply because technics for study of amino acids are readily available whereas work with more complex metabolites is less easily performed. There is a strong temptation to use amino acid analogues in attempts to discover amino acids essential for viral growth. However, effects on other amino acids or on other metabolites are usually not studied concurrently. The possibility of interconversion of the amino acids used must also be considered [Lien & Greenberg (136)]. More information might be obtained from a study of the metabolism of amino acids involved in any given system by use of one or more labelled components. This labelling may or may not be uniform and it is essential to establish the stability of the labelled atoms in the particular system used.

Although it may be difficult or impossible to obtain virus particles tagged with sufficient radioactive material to use for tracer studies, useful data can be gained by tracing other components provided the virus involved can be quantitated. The validity of this assumption is seen most readily in studies of bacteriophages which at present can be assayed more readily than any other virus.

In work with animal and plant viruses fruitful studies may be concerned with the relation of cell particulates to the processes of viral replication. In a discussion of the physical and chemical nature of cellular particulates Claude (137) states,

"A living cell, therefore is not a biochemical continuum but a composite entity, the sum of the interactions of associated elements which appear to be heterogeneous, if not autonomous. In this respect it is probable that, as the mechanisms of cellular synthesis are better understood, the concept of 'self-duplication' will be enlarged and extended to other components of the cell. In recent years, attention has been centered almost exclusively on the problem of reduplication of gene substance, although it is obvious that all the other essential cell structures are likewise reduplicated during cell growth, or at the time of cell division."

A solution to the problem of how mitochondria and microsomes are duplicated in cells may well lead directly to an understanding of the processes of viral duplication. The former contain much energy-rich phospholipids and nucleic acids both of which are likely substrates for viral activity. Only little work, some of which has been considered here, has been done thus far on possible cellular loci of viral activity. This would assume unusual importance if intermediate forms of virus which are not detectable extracellularly are active within cells, a situation which occurs in the bacteria-bacteriophage relationship.

Much more work may be expected on the effects of hormones on viral infections. Biologic effects such as increased or decreased susceptibility of hormone-treated hosts of the type reported by Southam & Babcock (138) eventually will be explained on a chemical basis.

It is generally assumed that various substances such as heavy metals or toxic chemicals such as azide mediate their inhibitory action by interference with one or more enzyme systems. Presumably host tissue enzymes are primarily involved as discussed by Bauer (139). Obviously, more than one enzyme and its substrate may be inhibited yet usually the activities of only one or few enzymes are studied. This difficulty emphasizes the need to characterize the substrates which are vitally necessary in the virus-host cell reaction.

The comment has been made that solution of the basic problems of viruses will be possible only as new biophysical and biochemical technics become available. Despite the small number of studies made so far use of the technics that already can be applied should permit major advances in our knowledge. The principal need is to work, work, work.

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VIRAL AND RICKETTSIAL TOXINS[1,2]

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Physicians frequently have recognized and have been impressed with the toxic appearance of patients during the acute phases of certain rickettsial and viral infections. Within the past few years, it definitely has been shown by convincing laboratory procedures that toxic substances are intimately associated with certain rickettsial and viral agents. These toxins are demonstrable only in preparations which are extremely rich in infectious organisms, and thus far it has not been possible to dissociate the toxins from the living infectious agents. This review will attempt to cover the representative reports concerning these agents which have accumulated during the past thirteen years.

RICKETTSIAL TOXINS

Murine (flea-borne) and epidemic (louse-borne) typhus.—Gildemeister & Haagen (1) in 1940 were the first to show that mice injected intraperitoneally with 0.5 to 1.0 cc. of 1:25 dilutions of yolk sac suspensions richly infected with murine typhus rickettsiae (*Rickettsia typhi*) died within 4 to 20 hr. with "cramp symptoms" caused by a toxin. The toxic activity was proportional to the number of rickettsiae observed in the infected yolk sacs. The toxin was quite labile since it was rapidly inactivated by the addition of formalin, by heating at 60°C. or by storing at +2°C. for seven days. Any treatment that killed the rickettsiae also inactivated the toxin. Normal human and rabbit sera did not neutralize the toxin, but sera obtained from recovered typhus patients as well as from vaccinated individuals neutralized the toxin. Retroplacental blood in the form of "Homoseran," shown to be rich in protective substances for measles and poliomyelitis, also neutralized the toxin. A few sera were encountered that protected mice against the rapid toxic death but permitted them to die a few days later from infection. It was also noted that rich rickettsial suspensions were toxic for chick embryos, causing them to die prematurely with relatively few rickettsiae present in the yolk sac. Somewhat similar observations were made shortly thereafter by Otto & Bickhardt (2), Kligler & Oleinik (3), and Bengtson, Topping & Henderson (4).

Kligler & Oleinik (3) reported that yolk sac suspensions of either louse-borne or flea-borne typhus strains, when lightly centrifuged and injected

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[1] The survey of literature pertaining to this review was completed in February, 1953.

[2] The following abbreviations are used in this chapter: LGV (lymphogranuloma venereum); MLD (minimum lethal dose); NDV (Newcastle disease virus); RDE (receptor-destroying enzyme); W.E.E. (western equine encephalomyelitis).

intradermally into rabbits, produced on the second or, more usually, the third day an indurated, inflamed nodule with a central necrotic area [Giroud test (5)]. They found an equivalent suspension, practically freed of rickettsiae by centrifuging in an anglehead centrifuge at 4500 to 5000 r.p.m. for 2 hr., still gave a typical skin reaction in rabbits even when diluted 20 to 40 times. When injected intraperitoneally into mice and rats, the same supernatant fluid, freed of rickettsiae by centrifugation, produced considerable enlargement of the spleen and liver by the third to the fifth day. No rickettsiae could be found in the tissues under these conditions. Furthermore, if partially purified rickettsial suspensions were frozen and thawed seven or eight times, the same reactions were induced in rabbits, rats, and mice, although no living rickettsiae could be demonstrated either by yolk sac culture or by animal inoculation. The authors suggested that the skin lesions induced in rabbits and the splenic enlargement of mice and rats were traceable to a toxic substance which was contained in the rickettsiae themselves, and which was still present in the supernatant fluid that had been freed of rickettsiae by centrifugation.

The toxic substance was quite heat labile. It was completely inactivated by heating at 56° to 60°C. for 30 min. and largely inactivated even at 50°C. A reduction in toxic strength took place after seven days in the ice box (10° to 12°C.). Shaking with diethyl ether for 30 min. completely inactivated the toxic substance both in the centrifuged supernatant fluid and in rickettsial suspensions. No toxic substance was present after removal of the ether.

Bengtson, Topping & Henderson (4) reported two strains of epidemic typhus (Breinl and Madrid #1) that killed 12 to 17 gm. mice within 1 to 6 hr. by injecting them intravenously with 0.5 cc. of 1:20 or 1:40 dilutions of richly infected yolk sac suspensions. The promptness of death was related to the amount of toxic substance injected. The animals showed prostration, convulsive movements, and labored respiration followed by death. Toxin was stable in buffered yolk sac suspensions at $+4$°C. for 72 hr. and in skimmed milk-yolk sac suspension at -70°C. for three months. Toxin was destroyed within 30 min. by heating to 60°C., by addition of formalin to 0.375 per cent concentration, or by addition of diethyl ether.

Using either centrifugation or filtration procedures, it was not possible to separate the toxic substance from the intact, living rickettsial bodies. Sera from vaccinated cases and from a patient convalescent from epidemic typhus neutralized the toxin to various degrees, but sera from normal individuals and from a typical Rocky Mountain spotted fever case failed to neutralize. Subsequent studies by Henderson (6), Henderson & Topping (7), and Topping, Bengtson & Henderson (8, 9) showed that while the toxin of epidemic typhus is quite labile and intimately associated with the intact living rickettsial cell, yet the toxoid prepared by treatment with formalin is remarkably stable and quite antigenic in inducing the production of antitoxin (antibody) in guinea pigs and man (9).

The studies of Topping, Henderson & Bengtson (4, 6, 7, 8, 9), who de-

scribed methods of standardizing the "toxic substance" of typhus rickettsiae and for estimating the amount of toxin neutralizing antibodies in immune sera, served as the basis of standardization of all typhus vaccine manufactured in the United States and licensed by the National Institutes of Health. The standard potency test may be defined as a mouse protection test which measures the toxin neutralizing value of sera obtained from guinea pigs vaccinated with typhus vaccine (10, 11).

Craigie and his associates developed a direct mouse test for the assay of typhus vaccines (12) and showed that mice injected with such vaccines were actively immunized against the toxic factors of murine or epidemic rickettsiae injected either intraperitoneally or intravenously. They furthermore showed that the antibodies induced by the toxoids (vaccines) against the toxins of epidemic or murine rickettsiae are type specific and that the heterologous type of vaccine confers no regular cross immunity to the toxic factor even when large doses of vaccine are used in the mouse antigenicity test. The immunity produced by small doses of vaccine was quite type specific and dependent upon the presence of a heat labile antigen that could be inactivated by heating at 56°C. for 45 min. (12).

Groupé & Donovick (13) likewise reported on the marked specificity of the epidemic and murine typhus toxins. Mice that received a single inoculation of epidemic or murine typhus vaccine and were challenged 14 days later by an intravenous injection of three to four lethal doses of toxin were protected against the homologous toxic substance only.

Routine comparative tests made on batches of typhus vaccine showed some correlation between complement fixation titers, with finished vaccine as antigen and sera of vaccinated guinea pigs as antibody, and the mouse protection test which measures the toxin neutralizing capacity of the sera of vaccinated guinea pigs (11). It may be interesting to note that while the immunizing antigen of typhus vaccine is quite heat labile, the complement fixing antigen is remarkably heat stable, being not only resistant, but even enhanced in its activity by boiling (14). Similar observations were made by Bedson for psittacosis (15).

Hamilton (16) investigated the specificity of epidemic and murine typhus toxins and likewise concluded that the antibodies neutralizing these toxins are immunologically distinct. In absorption tests, rickettsiae of the homologous type markedly reduced the toxin-neutralizing antibody titer of immune sera, whereas rickettsiae of the heterologous type failed to do so. Within the epidemic and murine rickettsial types, strain differences in the potency of the toxic factors were encountered, but specificity of types was retained. Hamilton (16) also noted that the neutralizing antibodies were more readily absorbed by rickettsiae than were the complement-fixing antibodies. Clarke & Fox (17) reported that mice injected intravenously with toxic rickettsial suspensions showed a marked reduction in blood volume and suggested that the toxicity of richly infected yolk sac suspensions is due to a factor that seriously alters vascular permeability. The blood of

affected mice showed an increased concentration of red blood cells but not of plasma proteins, which indicated a condition similar to shock. Administration of additional fluid (saline intraperitoneally) or of such drugs as epinephrine (Adrenalin) or Pyribenzamine failed to affect either the survival time or the hematocrit readings obtained on the treated mice. However, mice that received mixtures of rickettsia-infected yolk sac suspensions and homologous antiserum not only survived, but failed to show any blood changes.

Bovarnick & Snyder (18) used the mouse toxin test to estimate the concentration of viable rickettsiae present in suspensions used for metabolic studies. Partially purified suspensions of typhus rickettsiae showed metabolic activity in the presence of glutamate and pyruvate; the rate of oxygen uptake was directly proportional to the concentration of living rickettsiae.

Recently Neva & Snyder (19) reported that white rats were susceptible to the acute toxicity of typhus rickettsiae in a manner comparable to white mice. The results obtained on repeated titrations in mice and rats were reproducible. With the Breinl strain of epidemic typhus rickettsiae, 5 to 20 mouse LD_{50} doses were found to be equivalent to 1 LD_{50} dose for the rat. Thus, on a weight basis the LD_{50} dose of the Breinl strain was nearly identical for rats and mice. A strain of epidemic typhus isolated in Ankara, Turkey, and the relatively avirulent Spanish epidemic strain E (20) were found to be lethal for white rats in dosages equivalent to 10 LD_{50} mouse doses. Following injection, rickettsial toxicity became manifest in white rats in about 30 min. or later. The animals huddled together, showed labored breathing, cyanosis, and finally prostration. Death occurred within $1\frac{1}{2}$ to 4 hr. with the larger doses, and as late as 18 hr. nearer the LD_{50} limit. Hyperirritability and convulsive movements, which are commonly observed in white mice, were not noted in white rats. At autopsy the most constant and significant findings were limited to the small intestine. The visceral blood vessels, particularly those of the small intestine, were enlarged and congested with blood. Fluid, sometimes yellow and sometimes bloody in color, was found in the gut. With large, toxic doses, the mid-portion of the small bowel was often dark red in color and quite often hemorrhagic. Quite similar gross findings were observed in mice dying from toxin, although the changes apparently were not quite as prominent. It may be noted that Burrows (21), in a review of bacterial endotoxins, mentions that animals injected with a fatal dose of various bacterial endotoxins frequently show hemorrhage in the small intestine. Neva & Snyder (19) also found that white rats which survived sublethal doses of epidemic typhus toxin failed to show any subsequent signs of typhus infection, although complement-fixing antibodies were induced. On the contrary, rats that survived sublethal doses of murine typhus toxin subsequently died of infection 48 to 72 hr. later. However, rats were found to be approximately five times more resistant to the toxin of murine typhus than to epidemic typhus toxin. These authors also point out the importance of the species of animal used for the demonstration of toxin; cotton rats, for example, injected intracardially with 25 to 50 LD_{50} toxic

doses for mice failed to show any toxic effect but died from infection three to four days later.

Scrub typhus.—Smadel and his associates (22) demonstrated a toxin for mice with the Gilliam strain of *Rickettsia tsutsugamushi*. Mice injected intravenously with richly infected yolk sac suspensions developed dyspnea and weakness within 1 to 1½ hr., followed rapidly by prostration, cyanosis, convulsions, and death. Practically all deaths from toxin occurred within 3 hr. after injection. Mice that received sublethal toxic doses died from scrub typhus infection four to six days later. Infected yolk sacs never showed toxin titers higher than 1:20 dilution. The toxicity of a rickettsial suspension was generally decreased by freezing and storing at −70°C. or −20°C. for a few hours but was apparently unaffected by storage at 0°C. for 3 to 4 hr. These investigators were unable to demonstrate a toxin associated with the Imphal, Calcutta, Karp, Kostival, and Host 21 strains of *R. tsutsugamushi*. Specific homologous antiserum neutralized the toxin, but antisera prepared against heterologous strains of *R. tsutsugamushi*, as well as other rickettsial groups, failed to neutralize the toxin.

Miscellaneous.—Jackson & Smadel (23) reported failure to demonstrate any protective effects of adrenocorticotropic hormone (ACTH) or cortisone on the toxicity of *R. tsutsugamushi* or *R. prowazekii* for mice. Kass, Neva & Finland (24) likewise showed that ACTH did not reduce the acute toxic effect of rickettsial suspensions of murine or epidemic typhus for mice.

On the other hand, it has been demonstrated that aureomycin has a direct effect on rickettsial toxins, neutralizing them *in vitro* (25, 26). Chloramphenicol apparently lacks this property, whereas terramycin possesses it to a much less degree than aureomycin, being approximately one-tenth to one-fifteenth as active. It has been found that when suitable quantities of aureomycin are mixed with toxic suspensions of fully virulent, living rickettsiae and the mixtures are held for 20 to 30 sec. before intravenous injection into mice, the mice survive without showing signs of illness, whereas control mice similarly injected but with no active aureomycin in the mixture, die typically within a few hours. The capacity of aureomycin to neutralize toxin is diminished but not entirely destroyed by heating at 100°C. for 10 min. at pH 3.0 and is completely destroyed under the same conditions at pH 7.0. Neutralization of rickettsial toxins takes place almost immediately. The reaction is not rickettsiocidal since such nontoxic mixtures produce the same LD_{50} endpoints in chick embryo titrations as do toxic control suspensions. The reaction is quantitative since increasing quantities of aureomycin are required to neutralize increasing numbers of LD_{50} toxic doses of a single rickettsial strain. Different amounts of aureomycin are required to neutralize equivalent LD_{50} doses of different rickettsial and viral toxins, such as the Gilliam and Karp strains of *R. tsutsugamushi*, the Wilmington strain of *R. typhi*, the Breinl strain of *R. prowazekii*, the 6BC strain of *Miyagawanella psittacii* (psittacosis), the SF strain of *M. pneumoniae* (human pneumonitis) and the F99 strain of influenza type A. The toxins of scrub typhus are neu-

tralized by the least amount of aureomycin, 7.5 µg. for 2 LD_{60} toxic doses, whereas the viral toxins require the most. It was possible to demonstrate *in vivo* neutralization of the toxins of the Karp and Gilliam strains of *R. tsutsugamushi*. Thus 1.0 mg. of aureomycin HCl injected intravenously 30 to 60 sec. prior to 2 LD_{60} toxic doses completely protected all mice. Toxin followed by antibiotic was slightly less effective (80 to 90 per cent protection). Beneficial effect was still obtained when the antibiotic was given as late as 15 min. following injection of toxin. Attempts to obtain similar results with other viral and rickettsial toxins failed, presumably because too great a quantity of aureomycin is required.

Thus far, no toxins have been clearly demonstrated for spotted fever (*R. rickettsii*), Q fever (*Coxiella burnettii*), or rickettsialpox (*R. akari*). However, it should be noted that chick embryos inoculated with heavy concentrations of *R. rickettsii* die prematurely with very few, if any, rickettsiae present in the yolk sac tissues. These findings surely suggest a toxic effect (27). The same observation is true for *R. prowazekii* and *R. typhi* (27).

VIRAL TOXINS

Psittacosis-lymphogranuloma venereum group.—In 1943 Rake & Jones (28) demonstrated a toxic factor in yolk sacs heavily infected with the virus of lymphogranuloma venereum. The toxic factor was of relatively low titer since it was not present in the yolk sac in dilutions higher than 1:40 nor in infected yolk fluid higher than 1:2. Ten to 20 gm. mice injected intravenously with 0.5 ml. volumes of richly infected yolk sacs usually died within 4 to 24 hr.; convulsions usually preceded death. The toxic factor was associated with the bodies of the infectious agent since the two could not be separated by centrifugation or filtration procedures. Immune serum prepared in rabbits specifically neutralized the toxic factor. Subsequent studies demonstrated similar toxins to be present in yolk sac suspensions of the viruses of psittacosis (28), meningopneumonitis, mouse pneumonitis (29), and feline pneumonitis (30). The toxins of all the above agents are quite labile, being rapidly destroyed within a short time at 37°C. or by relatively low concentrations of formalin. The toxins are found in highest concentration in infected yolk sacs. They rapidly kill mice after intravenous and occasionally after intraperitoneal injection, but extremely large doses, corresponding to about 35 to 40 million infective doses, are necessary to produce toxic death. Each of the viruses produced a characteristic death curve when injected intravenously into mice, showing an initial interval during which toxic deaths occurred, a second period during which few or no deaths occurred, and a third period during which many of the remaining mice died of infection (30). Mouse pneumonitis toxin caused death very rapidly, within 1 to 4 hr. after injection, while feline pneumonitis toxin killed within 6 to 24 hr. Toxic deaths due to meningopneumonitis usually occurred within 12 hr. after injection. The curve for LGV[2] differed from that of the other three viruses mentioned above in that mice injected with this virus died only from the toxin and

showed no secondary rise in deaths due to infection. Yolk sacs infected with feline pneumonitis and meningopneumonitis killed mice from toxic effects in dilutions as high as 1:100, whereas much lesser amounts of toxin were found in yolk sacs infected with LGV (1:10) and mouse pneumonitis (1:5). The toxins of these agents were quite specific and could be neutralized only by homologous antiserums (30). Toxin-neutralizing antiserums were prepared by the injection of toxin or toxoid (formalinized material) into rabbits or chickens. Suspensions of chorioallantoic membranes and allantoic fluids from eggs heavily infected with feline pneumonitis virus were also found to be toxic for mice. The toxin could be neutralized by specific immune serum (31). Pathological studies carried out by Rake & Jones (29) on mice dying from LGV toxin showed congestion of the lungs in early deaths, and enlarged yellow livers in those dying at 20 hr. or later. Microscopically, the most constant features in deaths up to 9 hr. were scattered hemorrhages in the lungs, patches of edema fluid in the alveoli, and fibrin thrombi in the glomerular capillaries of the kidneys. The liver occasionally showed foci of marked necrosis and more frequently foci in which the cells appeared damaged and loaded with fat droplets, but were not yet necrotic. Mice dying at 15 to 24 hr. rarely showed hemorrhage or edema of the lung. Fibrin thrombi were not seen in the kidneys but occasionally tubular damage was seen. The most pronounced damage was observed in the liver. In almost all cases there were numerous foci of necrosis throughout the liver. These foci were often accompanied by hemorrhage, and those liver cells that were not necrotic usually showed marked cloudy swelling. Necrosis of the liver lobule was always midzonal. Mice dying from meningopneumonitis toxin showed symptoms very similar to those seen in LGV. In early deaths, up to 60 hr. after injection, the macroscopic appearance of the tissues was similar to that seen in LGV. The bright yellow livers suggested extensive necrosis. In later deaths macroscopic changes in the liver were quite striking because of regeneration of new liver lobules; in addition, pin-point, plum-colored areas appeared in the lungs, and the spleen, which often became large and soft, often showed yellowish grey areas. Mice that died within 24 hr. from feline pneumonitis toxin showed some edema in the lungs, cloudy swelling of the liver, and occasional scattered necrotic liver cells. Mice that died after two days showed many Kupffer cells distended with viral vesicles. Necrosis of liver cells was present but was of an entirely different character from that seen following intravenous inoculation with other agents of the psittacosis-LGV group. There was no midzonal necrosis, and only single cells or small lobules were involved. Both neutrophils and monocytes promptly invaded the necrotic tissue, and the areas became walled off to become abscess-like. By the third day many active giant cells were present in the pulp of the spleen, and some showed one or even two viral vesicles.

Rake & Hamre (32) demonstrated that sulfamerazine had no effect upon the early toxic deaths caused by any agent of the psittacosis-LGV group even when only borderline toxic doses were used. Deaths resulting from infec-

tion by LGV or mouse pneumonitis could be prevented by sulfonamide therapy but those due to feline pneumonitis could not be prevented.

It was possible to get passive transfer of protection against the toxins (29). Thus mice that received an intravenous injection of antiserum immediately before the administration of toxin intraperitoneally were protected. From their studies Rake & Jones (29) concluded that the toxins of the psittacosis-LGV group of agents appeared to fit into the general class of endotoxins.

In 1949 Roca-Garcia (33) described the isolation of a new virus called opossum virus A, from the Colombian opossums *Didelphis paraguayensis* and *Caluromys laniger*. Like other agents of the psittacosis-LGV group, this virus grew readily in the yolk sac tissue of the developing chick embryo and produced a toxin which killed mice which had been injected intravenously with heavily infected yolk sac suspensions. Mice that received 0.5 ml. of a 1:20 yolk sac dilution died within three hours. When greater dilutions were used deaths took place from 6 to 48 hr., with 45 per cent of the deaths taking place within 24 hr. The virus appeared to be more closely related to LGV than to any other member of the psittacosis-LGV group.

Manire & Meyer (34) tested 39 virus strains of the psittacosis-LGV group for their toxicity in mice. Twenty-two strains were of mammalian origin (human, cat, and mouse) while the remaining seventeen were of avian origin. Infected yolk sac suspensions, which were used as the source of toxin, were injected intravenously in white mice of a single strain. Seventeen of the twenty-two mammalian strains killed mice with a toxic factor in dilutions of 10 per cent yolk sac or greater within 24 hr. Two of the human strains (Louisiana and S-F pneumonitis) killed mice in a maximal dilution of 1:80 and were the most lethal of any strains tested. Five of the 17 strains isolated from birds failed to kill mice within 24 hr. with yolk sac suspensions of 1:10 or greater. The findings of Rake & Jones (28, 29) and Hamre & Rake (30, 31, 32) that meningopneumonitis and feline pneumonitis viruses showed two-phase death curves were confirmed. Manire & Meyer (34) found a similar two-phase death curve for the Louisiana and Illinois viruses. With the Louisiana virus toxic deaths occurred during the first 16 hr., followed by deaths due to infection starting at 48 hr. With the Illinois virus the peak period for toxic death was during the first 24 hr. and the peak period for infective deaths came at 96 to 120 hr. Mice that survived intravenous injection of the S-F group of viruses showed no signs of infection. All 15 virus strains of human origin showed some toxicity for mice.

In a second paper Manire & Meyer (35) reported on the use of aureomycin and penicillin in protecting mice against the toxins and infections due to various strains of psittacosis virus. Three groups of viruses were tested: the Louisiana and feline pneumonitis (Baker) strains, which showed two-phase death curves in mice; the S-F virus, which was toxic but not infectious intravenously for mice; and five viruses (P-Endo-1, H-SE, H-Mu 46, Pasadena parakeet, P-207) which were both toxic and infectious for mice by the intravenous route but produced single-phase death curves. The mice were

first injected intravenously with infected yolk sac suspension and then treated subcutaneously with aureomycin or penicillin. Aureomycin was given as 0.5 mg. of drug in 0.5 ml. of saline at 12-hr. intervals. Penicillin was given in dosage of 5000 units per day of procaine penicillin G in 0.1 ml. of beeswax and peanut oil suspension. Neither penicillin nor aureomycin had any effect on the toxic deaths caused by the Louisiana, S-F, and feline pneumonitis strains. However, deaths from infection resulting from the Louisiana and feline pneumonitis strains were prevented. Penicillin appeared to be less effective than aureomycin. Mice that survived Louisiana virus following aureomycin therapy were found to be carriers of the virus by subinoculation of their spleens into yolk sacs of embryonated eggs. Carriers were not found in aureomycin-treated mice that survived feline pneumonitis and S-F virus inoculation. S-F virus did not produce infection; thus no drug benefit was found.

Treated mice that received the weaker toxic strains (P-Endo-1, H-SE, H-Mu-46, P-207, and Pasadena parakeet strains) showed markedly reduced deaths apparently from toxin. Treated mice that survived injection with P-Endo-1, P-207, and Pasadena parakeet strains showed signs of carrier infection. Manire & Meyer (35) pointed out that the toxins of these virus agents differ from the bacterial endotoxins. The extreme lability of the viral toxins, which are destroyed in the incubator in a very short time, and their ready detoxification by formalin without loss of antigenicity are not generally considered characteristic of bacterial endotoxins. Manire & Meyer (35), together with Doerr (36), suggest that the toxins of the psittacosis-LGV viruses and of the rickettsiae are more nearly similar to the bacterial exotoxins.

In a third paper Manire & Meyer (37) reported their results on toxin neutralization tests in mice with 27 virus strains of the psittacosis group. Antisera were prepared in chickens against nine virus strains [Louisiana, S-F, H-SE, H-We, H-Mu-46, meningopneumonitis (Cal 10), P-Endo-1, P-Endo-3, and feline pneumonitis (Baker)] and tested against each of the 27 virus strains. On the basis of these tests the 27 virus strains were found to fall into six separate groups. Of these only the toxins and corresponding antisera of the Louisiana and feline pneumonitis strains were truly specific, reacting only in homologous mixtures. The remaining 25 virus strains showed a rough separation into four main groups which more or less overlapped in their toxin neutralization characteristics. Two of the groups apparently were new ones to be described. One of these was composed of a large number of viruses from numerous sources: man, pigeon, parakeet, duck, chicken, canary, and mouse. The second group consisted of four viruses all of pigeon origin.

Influenza.—In 1944 the Henles (38) first showed that neurological signs could be produced in mice by intracerebral inoculation of allantoic fluids containing active human or swine influenza virus. High titered infected allantoic fluids or virus concentrates prepared therefrom produced marked hyper-

irritability in practically all inoculated mice within 12 to 48 hr. When the mice were suspended by their tails, they showed marked tremor and clonic convulsions which could change suddenly into tonic convulsions, with death occurring in a large percentage of the animals within 24 to 72 hr. Preliminary histological examination showed definite meningo-encephalitic changes in the brain. The cerebral signs, which were shown to be caused only by active influenza virus, were produced by all strains of influenza tested: PR8, WS, Weiss, F-12, and F-99 strains of influenza A, Lee strain of influenza B, and a strain of swine influenza. The toxic cerebral symptoms could be prevented by neutralization of the virus suspensions with high titered immune sera. The neutralization test was quite specific, since the influenza A strains were neutralized only by anti-A sera, while the B strains were neutralized only by anti-B sera. Inactivation of the virus preparations by ultraviolet irradiation, heat, or formalin destroyed the toxic agent. High concentrations of irradiated virus, when mixed or injected simultaneously with active homologous or heterologous virus by the intracerebral route, showed evidence of interference in that there was a reduction in the incidence of convulsions as compared with that in control mice which received active virus only. The same neurological phenomena were elicited by influenza viruses maintained continuously by mouse lung passage, provided sufficient quantities of virus were present. The virus did not propagate in brain tissue, but actually decreased progressively in the original brain tissue, and attempts to pass the virus from brain to brain failed.

Evans & Rickard (39) demonstrated that injection of type A or B influenza virus into the rabbit eye produced corneal opacity without multiplication of the virus. The severe corneal damage induced was attributed to a toxic effect of the virus and not to infection. The toxic effect was readily neutralized by specific antiserum.

In subsequent studies (40, 41, 42), the Henles reported various strains of influenza virus to be toxic for mice when injected intraperitoneally or intravenously. Toxic deaths occurred within 8 to 96 hr. with lesions resembling in many respects those described for viruses of the psittacosis-LGV group (29). Severe damage was observed in the liver. The liver, which usually was of normal size with sharp edges, showed "a fine mottling of varying degree caused by small zones of normally colored liver tissue on a yellowish background of necrotic areas." Histologically, hyperemia and focal necrosis were seen. The spleen also was severely affected, usually being enlarged and dark red in color. Histological examination showed marked hyperemia and destruction of the Malpighian bodies. Certain of the more toxic virus strains (F-99, F-12, PR8, and WS) produced marked engorgement of the blood vessels of the intestines. The gut was edematous and contained bloody mucous material that varied in color from light to dark red. Occasionally clotted blood was observed in the stomach. The Lee and ES strains of influenza B as a rule did not produce intestinal reactions but did produce large amounts of pleural exudate, especially if the mice survived two or three days following

injection. The lungs showed engorgement of the blood vessels. With certain strains of virus, particularly the F-99, F-12, and PR8 strains, a second peak of deaths due to infection occurred six to eight days after injection.

The liver appeared to be the most severely affected by toxic action of the virus following intraperitoneal injection, but repeated attempts to pass the virus by liver tissue transfer failed. Virus concentration was highest in the peritoneal cavity shortly after primary injection of virus, and it decreased rapidly thereafter.

It was noted that strain differences of influenza virus, apparent in the intranasal neutralization tests, could also be demonstrated by neutralization of the toxic factor. Thus, the F-99 strain was neutralized only to a slight degree by the anti-PR8 serum, whereas the F-99 serum was equally effective against both F-99 and PR8 strains.

Injection into chick embryos of highly diluted inocula produced higher titers of virus, hemagglutinin, and toxicity in the allantoic fluids than did the use of more concentrated seed cultures. This was confirmed by Kempf & Harkness (43). Serial passage of various strains in high dilution quite often increased toxic activity (42). The toxic activity could not be separated from the infective property by such means as differential centrifugation and adsorption onto and elution from chicken red blood cells. However, the infectivity often reached its peak in 24 hr. when tests for toxicity were still negative. Maximal toxicity was usually not attained before 48 hr. Moreover, on heating, formalinization, and irradiation with ultraviolet light, the ability of the virus agent to propagate was lost at a faster rate than was the toxic property (42).

Hale & McKee (44) confirmed that the PR8 and Lee strains of influenza, although incapable of propagation in mouse brain tissue, produced a lethal toxic effect when injected into mice intracerebrally. All tests showed that the toxic agent was intimately associated with the virus agent itself.

Dudgeon and his associates (45) reported on their attempts to adapt influenza B viruses of the 1946 outbreak to mice. Four of the six strains which had had three or four passages in chick embryos produced extensive consolidation in the lungs of the first mice inoculated intranasally. The lesions were as extensive in mice killed after four days as in those killed after eight days. No mice died of infection. The extensive lung consolidation was never observed again by continuing passage of the virus strains in mice. A hamster inoculated with undiluted infected allantoic fluid similarly died with complete lung consolidation three days later, but attempts to produce a transmissible pneumonia in mice failed. The lesions thus produced were attributed to virus toxin.

In 1947 the Henles (46) reported that the various properties of influenza virus are affected at different rates by exposure to ultraviolet irradiation, and that the decrease in activity obtained followed in part single and in part multiple hit curves. The properties of the virus were affected in the following order: (a) ability to propagate; (b) toxic activity; (c) interfering property in

the chick embryo and the inhibitory effect on embryonic development; (d) hemagglutinating capacity; (e) complement fixing activity.

In 1948 the Henles (47) suggested the use of a toxin test to standardize the potency of influenza vaccines in a manner similar to that employed for the evaluation of typhus vaccines (4 to 11). Mice immunized with the PR8, WS, Melbourne, F-99, F-12, and Weiss strains of influenza A, the Lee and ES strains of influenza B, and the S-15 strain of swine influenza showed specific resistance to the toxic activity. It was not possible to separate the immunity against the toxic property (intravenous test) from the resistance to infection (intranasal test). Results indicated that the intravenous toxin test measured the same immune response as the intranasal infection test. The only apparent objection to using the intravenous toxin test was that certain influenza strains, such as Weiss strain A, were not good toxin producing strains.

Harris & Henle (48) demonstrated that rabbits injected intravenously with PR8 and F-99 strains A type and Lee strain type B showed a profound transitory lymphocytopenia. This reaction was a specific property of the influenza virus particle since it was neutralized only by type-specific immune serum. However, the lymphocytopenic effect was not caused by influenza toxin nor was it specific for influenza since other agents such as mumps virus, solutions of hemolytic streptococcal nucleoproteins, and heat killed pneumococci produced the same effect. The data suggested that nucleoproteins were responsible for the reaction.

Bennett, Wagner & LeQuire (49) reported that rabbits injected intravenously with chorioallantoic fluids infected with PR8 or Lee strains of influenza showed a febrile reaction beginning $1\frac{1}{2}$ to 2 hr. after injection, reached a peak of 3° to 4°F. above the baseline during the next 4 hr., and then gradually returned to normal. Doses as low as 0.025 ml. of allantoic fluid produced fever. Type specific immune serum prevented the febrile response. However, the febrile response was not dependent on the infectivity of the virus but seemed to be related to its adsorptive capacity. Heating at 56°C. for 30 min., which destroyed viral infectivity but allowed partial retention of hemagglutinating activity, did not destroy the capacity to produce a febrile reaction. Heating at 62°C. for 30 min., which destroyed hemagglutinins, also destroyed the pyrogenic factor. No data were presented concerning the possible relationship of the rabbit pyrogenic factor to the toxic factor for mice.

The observations on lymphocyte changes in rabbits by Harris & Henle (48) were confirmed by Wagner et al. (50), who further noted that the lymphopenia produced by the virus was paralleled by its pyrogenic effect. Similar observations were made for the "B" strain of Newcastle disease.

It was also found that rabbits that showed fever on the first intravenous injection of influenza virus were unresponsive to a second injection of virus 24 hr. later. This tolerance was demonstrated for heterologous as well as homologous strains in a pattern corresponding to the in vitro receptor gradient of Burnet, McCrea & Stone (51). The state of resistance to homologous

virus challenge was of short duration, complete return of susceptibility being evident at the time of maximal circulating antibody response. Filtrates of *Vibrio cholerae* and *Clostridium welchii* which contained receptor-destroying enzyme were also effective in minimizing the pyrogenic response to subsequent injection of virus (52).

Heating sufficiently to destroy the hemagglutinins also rendered the virus nonpyrogenic (50). The pyrogenic effect was neutralized by type specific antisera. Certain differences between the febrile response induced in rabbits by injection of viruses and bacterial pyrogens were noted. The lag period between injection and rise in temperature was longer with viruses than with bacterial pyrogens. Relatively low temperatures inactivated the fever producing capacity of viruses, whereas bacterial pyrogens withstand prolonged autoclaving. Viral pyrogens are specifically neutralized with immune sera, whereas bacterial pyrogens treated similarly are unaffected.

Kempf & Chang (53) found that 250 to 300 gm. male albino rats injected intravenously with PR8 influenza preparations showed a lowering of arterial blood pressure due to a toxic property of the virus. The hypotensive factor was intimately associated with the virus particle and was neutralized by type-specific immune sera prepared in ferrets. The factor which was destroyed only by heating at 56°C. for 16 hr. was much more stable than certain other viral properties, such as the hemagglutinins, infectivity for chick embryos, and toxicity for mice. A threshold concentration of approximately 8000 hemagglutinating units of virus was required to elicit the hypotensive response. Later studies (54) showed the hypotensive factor to be present in four other strains of influenza, the K-U, 23, FM-1, and Lee strains. The hypotensive activity for rats and the toxicity for mice was markedly diminished by storage at −70°C. for six months, whereas infectivity for chick embryos and chicken cell agglutinin (CCA) titers were well maintained. Intravenous injections of virus preparations that induced a marked drop in blood pressure produced no detectable changes either in the caliber or the rate of flow through the mesenteric blood vessels. The heart, however, showed changes within 2 min. after virus injection. Both ventricles appeared dilated, and the auricles and great veins were enlarged. The ventricular contractions of all the rats were reduced in amplitude to one-fourth that of the control animals, but the rate of ventricular contraction remained unchanged. Microscopically, the organs of the rats were normal, with the exception of congestion of the blood vessels of the myocardium. These findings were similar to the findings reported by Finland and his associates (55), who suggested that influenza virus has a direct toxic action on the cardiovascular system. In two cases of fatal human infection from which influenza A was isolated, extensive myocardial necrosis was observed.

Sugg (56) reported that the egg adapted CAM strain of virus, which showed little, if any, multiplication in mouse lung, produced death of mice from toxic action when introduced intranasally. The pulmonary toxic effect did not differ in gross appearance from that seen in the lungs of mice dead

from infection, but the toxic action could not be repeated by passing lung tissue suspensions to normal mice.

Later the same worker (57) reported that either the unadapted or adapted line of the CAM strain of virus killed mice from the same cause, that is, a toxic action on the affected cells following intranasal inoculation but, the time of death only was different. Large amounts of viral inoculum killed mice rapidly from toxic action without much multiplication of virus. On the other hand, smaller amounts of virus multiplied until a certain critical concentration of virus was reached; this then killed by the same toxic mechanism. This author suggests that the production of pulmonary lesions in mice inoculated intranasally with influenza virus depends to a great extent on the concentration of intracellular virus.

Greiff and his associates (58) demonstrated that allantoic fluids infected with influenza virus contained a toxic factor which profoundly modified the respiration of chick embryos. The toxin in concentrations of 0.5 ml. volumes or less of infected fluids had no effect on oxygen consumption. Injection of 0.75 to 2.0 ml. volumes strikingly increased the oxygen consumption of fertile eggs, whereas volumes of 2.0 ml. or more markedly depressed respiration. The toxin was slowly inactivated by heating at 56°C. but its activity was retained long after virus infectivity and hemagglutinating action were destroyed. In this respect it differed from the toxins of the rickettsiae and the viruses of the psittacosis-LGV group.

Kempf & Harkness (43) found the ferret *Putorius putorius* to be susceptible to the toxin of influenza. An influenza preparation injected on the basis of 154 hemagglutinating units per gm. body weight killed six of eight ferrets within 18 hr., whereas 46.5 hemagglutinating units were on the borderline of toxicity. Mice were more susceptible to the toxin than ferrets, since 53 hemagglutinating units per gm. body weight killed mice. Ferrets dead from toxin within 24 hr. showed no gross changes in the tissues, but microscopically congestion and infiltration with polymorphonuclear cells were observed in the liver, spleen, and adrenal glands. They found the toxicity of allantoic fluids to be stable for three months at −30° to −60°C., but not stable at 4°C. Some evidence was obtained that the toxin is different from the infective component, since on two occasions virus that had been stored at −60°C. for six months had lost its capacity to produce the toxic factor when inoculated into embryonated eggs, whereas the capacity to produce hemagglutination and infection was retained. In another instance the capacity to produce toxin was lost on serial passage in eggs.

Bernkopf (59) inoculated deembryonated eggs with different dilutions of virus and observed that in the presence of high concentrations of virus little or no evidence of multiplication took place as determined by infectious titer, while the hemagglutinin titers showed a consistent rise apparently independent of the quantity of active virus present. The more dilute inocula induced a corresponding rise in both infectivity and hemagglutinating titers.

The hemagglutinin is apparently formed in the chorioallantoic membrane.

It is able to interfere with the multiplication of active virus in eggs and mice but is incapable of self-propagation. Its appearance apparently depends upon the presence of large amounts of active virus. The hemagglutinin apparently has a shorter lag phase than fully active virus from which it cannot be differentiated in hemagglutination-inhibition tests. On intracerebral inoculation of mice the hemagglutinin factor showed a low toxicity for mice. Bernkopf considered the hemagglutinin to be an "incomplete" or "immature" form of virus (59).

Schlesinger (60) reported that mouse brain, which previously was considered to be insusceptible to infection with nonneurotropic strains of influenza virus, supported a single cycle of viral reproduction in which hemagglutinins and complement fixing antigen increased without an accompanying rise in infectivity titer. On the contrary, the infective titer decreased progressively in mouse brain. The newly formed "incomplete" virus was noninfectious, but was produced only if the intracerebral inoculum contained fully infectious virus. The yield of "incomplete" virus was proportional to the amount of infectious virus inoculated. The constant period preceding a rise in hemagglutinin titer was longer for the Lee strain B type than for the PR8 strain A type in both mouse brain and allantoic membrane. Schlesinger suggested that the demonstration of "incomplete" virus reproduction in mouse brain might throw new light on the mechanism of toxicity of nonneurotropic influenza strains. Since it is probable that the integrity of infected cells is equally impaired by the production of either complete or "incomplete" virus, the toxic phenomenon might be in fact a manifestation of a bona fide virus infection of the "incomplete" type.

McKee (61) reported that nontoxic virus could be reproduced repeatedly by injection of large doses of undiluted, mouse unadapted virus into the allantoic sac of the chick embryo. The use of mouse adapted virus apparently would not allow nontoxic virus to be produced. The nontoxic virus appeared to be as good an immunizing antigen as the toxic type. The growth rates of the two types of virus were not the same; the peak concentration of the toxic type of virus was reached in 16 hr., whereas the nontoxic type peak titer was reached in 22 hr. with an inoculum 10,000 fold less.

Wagner (62) used the WS and PR8 strains of influenza A and the Lee strain B type to study the altered susceptibility of the mouse to the neurotoxic action of influenza virus. Living Lee virus was considerably more effective in producing tolerance to the neurotoxic action of WS virus than to homologous challenge. Conversely, the neurotoxic action of Lee virus was as readily inhibited by a previous injection of WS virus as it was by the homologous strain. The degree of refractoriness induced by a preliminary injection of WS virus was of the same order for neurotoxic challenge with either WS or Lee virus. Lee virus heated at 56°C. for 1 hr. lost considerable protective action against challenge with homologous virus, but showed a less marked reduction in tolerance to WS virus challenge. Intracerebral inoculation of 0.03 ml. undiluted crude *V. cholerae* culture filtrate completely

abolished the neurotoxic response to 5 LD_{50} challenge of Lee, WS, or PR8 viruses given 25 hr. later. A greater concentration of crude cholera filtrate was required to produce neurotoxic tolerance against challenge with Lee virus than for the WS or PR8 strains. Kass and associates (63) reported that aureomycin or terramycin, administered orally or subcutaneously, did not increase the resistance of mice or rats to the acute toxic action of influenza A virus.

Fong & Bernal (64) reported that PR8 influenza virus treated *in vitro* with sulfur mustard or chlorethylamine derivatives showed inactivation of viral toxin and complete or nearly complete destruction of viral infectivity. The intensity of inactivation was a function of the concentration of mustard or its derivatives. The 5×10^{-3} and 5×10^{-4} molar solutions of mustards (except methyl mustard) brought about the greatest degree of inactivation of toxin and infective properties. The rate of destruction of these properties was a rapid one and appeared to be completed within 30 min. at 25°C.

Newcastle disease virus.—Burnet (65) was the first to report that mice given high titered Newcastle disease virus intranasally, followed by a second inoculation of virus or saline 24 hr. later, developed pneumonic symptoms somewhat similar to those produced by influenza virus. Some of the inoculated mice died with complete lung consolidation three to four days after the second inoculation, whereas others showed variable degrees of consolidation. Macroscopically there was nothing to distinguish the consolidated lung areas from those produced by influenza virus. Virus could be reisolated from lungs removed two to five days after the second inoculation, but there was little multiplication of virus and Burnet was unable to transfer the infection directly from mouse lung to mouse lung.

Hanson, Upton & Brandly (66) essentially confirmed the findings of Burnet (65) in that undiluted allantoic fluids infected with NDV[2] when introduced into the nares of partially anesthetized weanling mice sometimes produced pneumonia and death, and that it was not possible to transmit infection by lung to lung passage. The pneumopathic activity of NDV varied greatly according to the virus strain, some strains being highly lethal and others producing little, if any, lung involvement. Varying degrees of pneumopathogenicity for mice were demonstrated among 18 NDV strains. Some produced extensive pneumonia and death of 50 per cent of mice within 96 hr., whereas other strains produced no observable response whatsoever. The degree of pneumopathogenicity was not related to the degree of neurotoxicity, nor was it positively associated with the lethality of the strain for chickens. The pneumopathic property of the virus for the mouse was apparently less stable than the infectivity for the developing chick embryo.

Ginsberg (67) reported that the toxic factor of NDV is not confined to a single strain, although different strains vary in their toxin producing capacity. Thus when equal infective concentrations of virus were used, the Hickman strain of American origin was more toxic and produced better

lung lesions than did an Australian strain. Infected allantoic fluids containing 10^9 embryo infectious doses produced extensive pneumonia and death in the majority of mice, but the virus did not multiply sufficiently to maintain itself on serial passage. The second lung passage showed $10^{1.5}$ embryo doses, whereas the third lung passage was completely noninfectious. No multiplication of virus occurred in the lung. Thus at two days after inoculation there was 99 per cent less virus present than at five min. after inoculation, yet 40 per cent of the lung tissue was consolidated. Maximum pneumonia in mice was observed on the third day when one-third of all mice were dead and showed complete pulmonary consolidation. At this time when pulmonary lesions were most extensive, less than 0.1 per cent of the original virus could be found. A marked decrease in the amount of lung tissue involved and resolution of the lesions occurred thereafter.

Ginsberg was unable to duplicate the findings of Schlesinger with influenza virus (60). He could find no evidence that NDV produces "immature" or noninfectious viral particles that could still be revealed by hemagglutination or complement fixation techniques. NDV particles could not be readily dissociated from lung tissues, even after long periods of incubation in the presence of large quantities of RDE.[2] Extensive pulmonary consolidation of mice was produced only by a high concentration of virus. Allantoic fluid showing an embryo titer of $10^{9.3}$ killed mice in a dilution of 1:16 and produced maximal pulmonary lesions on the fourth day in titers as high as 1:64. Mice 20 weeks old or even older were just as susceptible as were mice three weeks old or newly weaned. The toxicity of NDV apparently could be demonstrated only by intranasal inoculation. The pulmonary lesions produced by NDV appeared indistinguishable from those caused by influenza virus (68) or by pneumonia virus of mice (69). Gross and microscopic examinations of lungs showed epithelial degeneration with eventual sloughing, interstitial infiltration of mononuclear cells and red blood cells, atelectasis, and marked pulmonary edema. Intranasal inoculation of RDE 6 hr. before administration of virus completely inhibited production of pulmonary lesions, whereas heated RDE and broth had no effect. RDE apparently restricts the association of NDV with susceptible cells just as has been observed for influenza. Injection of NDV immune serum before virus inoculation or at the same time completely prevented pulmonary consolidation but lesions still occurred when immune serum was injected intranasally 5 min. after NDV, although pneumonic lesions were significantly limited in their extent. Immune serum given 15 min. after NDV was still beneficial, but given at the end of 30 min. was ineffective. Influenza A virus, even though unadapted to mouse lung, multiplied extensively and interfered markedly with the production of pulmonary consolidation and death by NDV.

Davenport (70, 71) likewise reported NDV to be toxic for mice. The pathogenicity of the virus suspension was found to be related to the amount of virus given. If multiple inoculations of 0.1 ml. volume were used, all mice

died within 24 hr. with complete consolidation of their lungs. Infected allantoic fluids diluted tenfold produced no lung lesions. Inactivation of the virus by heating at 56°C. for 30 min. or by ultraviolet irradiation destroyed the pathogenicity of the suspensions, even though the capacity to cause hemagglutination was retained in the irradiated preparations. Virus suspensions that were concentrated by centrifugation or erythrocyte adsorption were pathogenic. The toxicity was specifically neutralized by immune serum. NDV suspensions were found to be toxic by intranasal inoculation but not when given intracerebrally or intraperitoneally. Intravenous inoculation was not tried. The data suggested that it was most unlikely that the toxic lung lesions produced by NDV were related to an incomplete cycle of multiplication of the virus. The pulmonary lesions observed with NDV were grossly indistinguishable from those seen with adapted and unadapted strains of influenza virus given intranasally. However, Davenport's microscopic findings differed from those of Ginsberg (67) in that NDV apparently did not produce necrosis and desquamation of the epithelial lining of the bronchi and bronchioles, which is seen regularly with influenza viruses. With NDV the lesions consisted essentially of a peribronchial and peribronchiolar interstitial pneumonia. Attempts to protect mice from the toxic effects of NDV suspensions by active immunization were not successful. Attempts to protect mice by vaccines administered intranasally were also unsuccessful. Mice were protected, however, by instillation of immune serum a few minutes before virus was given. Pulmonary toxicity was not abolished if immune serum was given as early as 5 min. after virus. The results showed that NDV was promptly adsorbed to the cells of the respiratory tract and promptly became inaccessible to the neutralizing effects of specific immune serum.

Liu & Bang (72) found a marked difference in the susceptibility of different strains of mice to pneumonia following intranasal instillation of NDV. Five strains of mice, all secured from Jackson Memorial Laboratory, Bar Harbor, Maine, were used, namely, the C strain inbred albino mouse, the C_{57} black inbred strain, the C_3H inbred strain, the dba line 1 and the dba line 2. Of these the dba line 2 and the C_3H showed the highest incidence of pneumonia while the other strains showed very few deaths from pneumonia. In addition they found encephalitis to occur usually 5 to 29 days after intranasal inoculation of NDV. The dba_1 mice were more liable to develop encephalitis following intranasal instillation than the C_3H or Swiss mice. These differences in susceptibility to NDV encephalitis were revealed only following intranasal inoculation. Intracerebral inoculation showed all strains of mice more or less equally susceptible. Younger mice showed a higher incidence of encephalitis following intranasal inoculation.

In our laboratory, Markham (73) found that freshly harvested allantoic fluids infected with NDV killed 10 to 12 gm. Swiss albino mice in 24 to 72 hr. when injected intravenously in 1 cc. doses. Pneumonic lesions were not observed. The cause of death appeared to be a hemorrhagic enteritis since

the small intestine was usually filled with what seemed to be altered blood. Dba mice apparently were not susceptible to the toxic effect. Fluids that had been frozen and thawed were no longer toxic and the toxin could not be demonstrated in fluids diluted more than 1:4. The lethal property of the NDV infected fluids was specifically inhibited by NDV immune chicken serum injected intraperitoneally 24 hr. prior to the intravenous inoculation of toxic fluids. Twenty-six strains of NDV were tested for toxicity. Their hemagglutinin titers ranged from 1:800 to 1:3200 and LD_{50} titers determined by chick embryo titrations ranged from $10^{-7.0}$ to $10^{-8.5}$. No close correlation was found between their toxicity for mice and their hemagglutination titers, their LD_{50} titers for chick embryos or their virulence as determined by intranasal or wing-web inoculation of 7-day-old susceptible chicks.

Mumps virus.—Bolin, Anderson & Leymaster (74) reported the development of a characteristic corneal reaction after the injection of relatively large amounts of mumps virus into the guinea pig's eye. The lesion induced apparently was caused by direct action of the virus on the endothelial cells of the inner surface of the cornea and was similar to that induced by influenza virus in the cornea of the rabbit's eye (39). Subsequent to intraocular inoculation, 99 per cent of the virus disappeared from the eye within 48 hr., following which virus grew or persisted within the ocular tissues for at least eight days. Powell & Culbertson (75) and Cabasso (76), to the contrary, were unable to detect any evidence of toxin associated with mumps virus.

Eastern and western equine encephalomyelitis.—Evans & Bolin (77) reported that eastern (E.E.E.) and western equine encephalomyelitis (W.E.E.) viruses when inoculated into the rabbit's eye produced a corneal opacity similar to the toxic reaction demonstrated for influenza (39) and mumps viruses (74). After injection of more than 300,000 mouse minimal lethal doses of virus into the anterior chamber of the rabbit eye, the aqueous humor contained considerable amounts of virus at 24 hr. but little or none at 48 hr. As in the experiments with influenza, large doses of equine virus (approximately a million MLD[2] for mice) had to be injected in order to produce the corneal reaction. Limited growth of virus might occur, but it was not a significant factor in the reaction.

Fastier (78) demonstrated that young mice (10 to 15 gm.) inoculated intravenously with high concentrations of mouse brain or chick embryo tissue infected with W.E.E.[2] died regularly within an accurately predictable time with characteristic toxic symptoms. Following intravenous inoculation with high concentrations of virus, mice remained well until about four hr. prior to death. Then the animals became hunched with ruffled fur and showed irregular, shallow breathing. Death was not accompanied by the muscular spasms and ataxia so typical of active virus infection. At autopsy the most obvious finding was the appearance of the liver, which was soft and hyperaemic with well demarcated areas of necrosis. Although congested, the spleen was not enlarged and gross necrotic lesions were generally absent. The

remaining organs appeared normal. Fifty per cent suspensions of infected chick embryo prepared either in serum-saline or in buffered saline showed no appreciable loss in toxicity when stored at 4°C. for four weeks. Heating at 56°C. for 30 min. destroyed the toxic properties of W.E.E. virus for both mice and rabbits, a procedure which greatly reduced but did not destroy infectivity. Rabbits inoculated intravenously showed a febrile reaction and associated leucopenia. Both the toxin and virus infectivity were specifically neutralized with immune serum.

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ANTIBIOTICS[1]

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During the year 1952, a great many new antibiotics were described and several were introduced for clinical investigation or use. It is fortunate that one of these, erythromycin, was found to be useful for the treatment of the rising number of infections caused by staphylococci resistant to most of the other antibiotics. Staphylococci have shown such an ability to become resistant to antibiotics and to cause superinfections that the future outlook for patients who have infections with these organisms is almost gloomy. New and potent antibiotics will have to be found and produced. It is evident that the field is and will continue to be in a dynamic state.

PENICILLIN

Veneral disease.—It is now nine years since penicillin was first used for the treatment of syphilis, and it is evident that it far surpasses any previously used antisyphilitic remedy when appraised from the therapeutic, economic, technical, toxic, or prophylactic aspects (1). An extensive review of the subject has been prepared by Beerman *et al.* (2).

For the treatment of early syphilis, schedules of 2 or 4 weekly injections of 1,200,000 units of procaine penicillin in oil containing aluminum monostearate resulted in retreatment rates which were not significantly greater than those observed following larger total doses administered at shorter intervals (3). Parkhurst and his co-workers (4) administered procaine penicillin G in oil with 2 per cent aluminum monostearate in two series of cases of secondary syphilis in which the diagnosis was proved by microscopic examination with darkfield illumination. In the first series, which consisted of 60 cases, the patients were treated by the one-injection method, that is, by the injection of 600,000 units of the preparation of penicillin at each of four sites. In the second series, which consisted of 86 cases, the patients were treated by the two-injection method, that is, by injecting 600,000 units of the same preparation of penicillin at each of two sites and by repeating the injection at these sites ninety-six hours later. After the patients had been observed for from twelve to fifteen months after the completion of treatment, the results were found to be successful in 88.2 per cent of the 60 cases in the first series and in 89.5 per cent of the 86 cases in the second series. It was evident, therefore, that one-injection and two-injection methods of treatment were equally effective. Similar dramatic results have been obtained with a single-injection schedule of repository penicillin (5) in the treatment of pinta, a nonvenereal treponematosis prevalent in Mexico.

[1] The survey of the literature pertaining to this review was concluded in January, 1953.

Penicillin given by mouth in doses of 250,000 units, within a few hours after exposure has been shown to be nearly 100 per cent effective in preventing gonorrhea (6). Its efficacy has remained unchanged after three years of use, and resistant forms of gonococci have not been encountered.

The hydriodide of diethylaminoethyl ester of penicillin G.—Because of its affinity for certain organs or tissues, there has recently been considerable interest in the hydriodide of diethylaminoethyl ester of penicillin G. This has been distributed under the trade names "neopenil," "leocillin," and "estopen." Various workers have found that, following administration of the ester, the concentration of penicillin in the lungs, lymphatic tissue, bronchial secretions, cerebrospinal fluid, and brain tissue of guinea pigs or humans is higher than it is after the administration of other forms of penicillin (7 to 13). Rhodes and his co-workers (14), however, said that the ester is microbiologically inactive and that active penicillin is obtained through hydrolysis. The question is raised as to how much of the total penicillin is in an active form.

An answer to this question appears to have been given by the results of some careful studies by Hallas-Moller and his associates (15). By using the usual analytical technics they found, as had previous workers, that the ester possessed an affinity for the lung. These methods, however, did not distinguish the content of ester and free penicillin in tissue. Since the unhydrolyzed ester has no antibiotic activity, analytical methods to determine only the amount of active penicillin present in blood or tissue were then employed. By using guinea pigs, it was found that after the injection of comparable amounts of ester and of procaine penicillin the active concentrations of penicillin found in the lungs and plasma after various intervals of time were somewhat higher when procaine penicillin was administered. Pulmonary tissue and red corpuscles of guinea pigs were found to absorb considerable amounts of the ester (60 per cent) which could not be recovered. These investigators concluded that even though the ester has an affinity for organs it is not capable of producing as high a concentration of active penicillin as is procaine penicillin. If this is true in human beings, this drug could not be expected to be as effective therapeutically as other types of penicillin.

STREPTOMYCIN

Treatment of tuberculosis.—The numerous papers which continue to appear attest to the importance of streptomycin in the treatment of tuberculosis. Because of the development of resistance of the tubercle bacillus to the drug when used alone, combinations of other chemotherapeutic agents with streptomycin are now being used with greater success. For the treatment of pulmonary tuberculosis streptomycin given intramuscularly two or three times each week and *p*-aminosalicylic acid given orally each day are a favored regimen (16 to 20). Viomycin has some toxic properties, but has been used for short-term treatment in combination with streptomycin or *p*-aminosalicylic acid.

The combination of terramycin and streptomycin compares favorably with streptomycin and *p*-aminosalicylic acid from the standpoint of therapeutic effectiveness and prevention of drug resistance (21). Isonicotinic acid hydrazide and certain other derivatives of nicotinic acid in combination with streptomycin are currently under investigation and appear promising (16). It has been reported that *in vitro* inhibition tests have revealed that there is a synergistic effect of mixtures of streptomycin and isonicotinic acid hydrazide both for streptomycin-sensitive and streptomycin-resistant strains of *Mycobacterium tuberculosis* (22).

It is evident that steady progress is being made in the chemotherapy of tuberculosis. It has become clear that tuberculosis should always be treated with drugs in combination, never with a single drug, thus greatly diminishing the likelihood that the organism will become resistant to chemotherapy (23, 24).

Toxicity.—Dihydrostreptomycin came into wide use because there was a much lower incidence of vertigo from disturbance of the labyrinthine mechanism associated with its use than with streptomycin. It has become evident, however, that dihydrostreptomycin given in daily doses for long periods may produce an even more distressing side effect, that is, impairment of hearing which is permanent (25 to 27). These toxic effects are prone to occur in cases in which the antibiotic has been given daily over long periods, such as in the treatment of tuberculous meningitis (28), and are rarely seen now that most patients with tuberculosis are being treated with combinations of drugs. In this method of treatment, streptomycin or dihydrostreptomycin is given in moderate doses and only two times a week (29).

Miscellanea.—Mitchell and his co-workers (30) found that streptomycin sulfate applied to the stems of bean seedlings was absorbed and transferred upward in sufficient amounts to suppress development of the halo-blight organism in the first, second and trifoliate leaves.

CHLORAMPHENICOL

Toxic effects.—Until recently, chloramphenicol has been considered to be a drug of remarkably low toxicity with relatively few side reactions (31). During 1952, however, a large number of reports have made it clear that the use of chloramphenicol may be followed by aplastic anemia, leukopenia, thrombocytopenia, granulocytopenia, agranulocytosis, and certain other reactions. In a considerable number of cases, the aplastic anemia has ended fatally.

Chloramphenicol contains a nitrobenzene radical, and it is known that drugs containing this structure may cause bone-marrow depression (32). A few early reports suggested that chloramphenicol might have a depressing effect on the bone marrow (33 to 36). It was not until recently, however, after several years of employment of this drug, that the full danger of its indiscriminate use has been realized. Lewis and his associates (37) recently reported the results of a study of 539 cases of blood dyscrasias by the Food

and Drug Administration to evaluate the magnitude of the problem. In 55 of the 539 cases, chloramphenicol was the only therapeutic agent that might have been responsible for the blood dyscrasia. In 44 of the 55 cases, the blood dyscrasia was aplastic anemia. Death occurred in 23 of the 44 cases of aplastic anemia.

Chloramphenicol is still the only chemotherapeutic agent that is effective against typhoid fever and certain other Salmonella infections, and it should be used for the treatment of such infections. The number of cases in which blood dyscrasias follow its use are only a minute fraction of the thousands of cases in which it has been employed. This potential danger, however, makes it imperative that the drug should be given only under medical supervision, that it should not be administered for minor infections, and that its long-continued or intermittent use should be avoided.

Chloromycetin palmitate.—An ester of chloramphenicol, chloromycetin palmitate, has been introduced for oral use (38, 39). Since powdered chloramphenicol crystals are very bitter, the oral administration of the drug to small children or other persons unable to swallow a capsule has been difficult. The palmitate is palatable. It apparently is hydrolyzed in the small intestine with the formation of chloramphenicol, after which the chloramphenicol is absorbed into the body and metabolized as usual.

AUREOMYCIN AND TERRAMYCIN

Structure.—It has been established that the structure of aureomycin and that of terramycin are very similar. These antibiotics are isomorphic and differ chemically only in the replacement of a hydroxyl group in terramycin by a chlorine group in aureomycin (40 to 42). This may explain the similarity in their spectrum of antibiotic activity. It is also consistent with the fact that organisms which become resistant to one concomitantly become resistant to the other (43). Two cases of crossed fixed drug eruption caused by aureomycin and terramycin have been reported (44, 45). These antibiotics are so similar that sensitization to one leads to sensitization to the other.

Administration.—Aureomycin calcium caseinate, a preparation designed to decrease the irritating effect of aureomycin on the gastrointestinal tract, has been studied by Manning & Wellman (46). The caseinate was found to be followed by much less irritation than regular aureomycin but yielded a comparable concentration in the blood. Siegel and his associates (47) studied the results of the rectal administration of aureomycin in cases in which the patients were children. The drug was poorly absorbed, and there was local mucosal irritation. They concluded that rectal administration of aureomycin is an unsatisfactory method of treatment. Since it is difficult to attain satisfactory concentration of aureomycin in the cerebrospinal fluid by oral administration, the antibiotic can be freshly dissolved in a suitable buffer to produce a neutral solution which may be administered intrathecally (48).

Treatment of bacterial infections.—A large number of papers continue to attest to the effectiveness of aureomycin and terramycin in the treatment of a wide variety of bacterial infections. In cases of surgical infections and peritonitis, the results of treatment with these antibiotics generally have been good (49 to 51). Reports on the use of these antibiotics in the treatment of brucellosis continue to appear (52, 53). It is evident that, when given alone, they do not cure brucellosis and that remissions resulting from their use are followed by a high incidence of relapses. Good results have followed the use of aureomycin or terramycin in cases of granuloma inguinale, chancroid, and plague (54 to 56), Subsequent to streptococcic infections, aureomycin has been used effectively in the prevention of rheumatic fever (57). When employed alone, terramycin was not effective in the treatment of streptococcic subacute bacterial endocarditis (58). A more bactericidal action generally is necessary for the cure of this disease. Aureomycin and terramycin have been reported to be useful in the treatment of pertussis (59 to 62). Because of the variable course of this disease, the results of therapy are difficult to evaluate. A number of papers confirm previous reports of the activity of these two antibiotics against spirochetal infections, including syphilis, frambesia, and *Leptospira* and *Borrelia* infections (63 to 67).

Terramycin was used successfully in the treatment of *Listeria* meningitis (68). Cannon & Leopold (69) found it effective when applied topically in cases of experimental keratitis due to a variety of gram-positive and gram-negative organisms. Aureomycin and terramycin were effective in preventing death in cases of experimental infections with seven different species of clostridia (70). In an epidemic of bacillary dysentery in Korea, aureomycin and terramycin, as well as chloramphenicol, were highly effective in clearing stools rapidly of *Shigella* (71).

Miscellanea.—Aureomycin has been used by Hesseltine and his associates (72) as a selective agent in the isolation of yeasts from corn-steep liquor or from soil. That aureomycin might have a profound effect on tissue metabolism is indicated by *in vitro* studies of Van Meter *et al.* (73), who found that it caused inhibition of oxidative phosphorylation in animal tissue.

Complications of therapy.—The increased importance of *Proteus* and *Pseudomonas* in causing infections following or associated with the use of antibiotics has been noted by Yow (74). The possible seriousness of giving the broad-spectrum antibiotics prophylactically must be considered, since, if an infection with a nonsensitive organism does occur in the course of such therapy, it is likely to present a difficult therapeutic problem. McCurdy & Neter (75) found that in infants the use of penicillin in conjunction with a broad-spectrum antibiotic was followed frequently by the appearance of a predominant gram-negative, bacillary aerobic flora in the upper part of the respiratory tract. Jackson and his co-workers (76) reported that death occurred in 7 of 91 cases in which pneumonia was treated with terramycin. In 4 of the 7 cases, the patients had severe superinfections with *Micrococcus pyogenes*, which contributed to or were responsible for the fatal outcome.

A number of reports have suggested that the irritative effects on the mucous membranes associated with administration of aureomycin and terramycin are the result of overgrowth of *Candida albicans* following elimination of the normal bacterial flora. It also has been suggested that the antibiotics actually stimulate directly the growth of *Candida* and other fungi. Seligmann (77) reported that suspensions of *Candida* injected intraperitoneally into mice were nonpathogenic, and that solutions of aureomycin injected by the same route were nontoxic, but that injection of the mixture was fatal. The virulence-enhancing activity of aureomycin was destroyed on heating. Lipnik and his co-workers (78) and Kligman (79) have studied the problem of fungous infections occurring in conjunction with treatment with antibiotics. It was found that aureomycin, chloromycetin, terramycin, and penicillin did not stimulate the *in vitro* growth of *C. albicans*. The *in vitro* enhancement of growth of *Candida* by material obtained from aureomycin capsules was found to be due to the inert phosphate in such capsules. There was no potentiation of mycotic disease in animals with experimental moniliasis, blastomycosis, histoplasmosis, coccidioidomycosis, or sporotrichosis by the wide-spectrum antibiotics. It was felt that the diagnosis of complicating moniliasis in many of the reported cases probably was not justified. The isolation of *C. albicans* in the presence of side reactions such as glossitis or stomatitis was not equivalent to a diagnosis of moniliasis. Paine (80) studied the influence of aureomycin, chloramphenicol, terramycin, penicillin, and streptomycin on the *in vitro* growth of yeast and monilia, and likewise found that they had little effect.

Anal and rectal irritation (81, 82) and vulvovaginitis (83) have been reported to have occurred after the use of the broad-spectrum antibiotics. Much more serious may be the colitis which may follow the use of aureomycin and terramycin (84). Reiner *et al.* (85) noted a sudden increase in the number of cases of pseudomembranous ulcerative colitis in which necropsy was performed, and they suggested that it was related to the use of aureomycin or, to the use of chloramphenicol in one case. Jackson and his co-workers (76), in reviewing the results of the treatment of pneumonia with terramycin, noted that gastrointestinal symptoms from terramycin were frequent and often severe, and that in cases of diarrhea induced by terramycin the fecal flora was often replaced by pathogenic staphylococci. These staphylococcal infections of the intestinal tract added considerably to the morbidity in many of the cases in which the patients recovered.

Janbon and his associates (86, 87) studied the digestive disturbances occurring in the course of treatment with terramycin. They concluded that such disturbances were due to an imbalance of the fecal flora, *Escherichia coli* being eliminated and *Proteus* growing in great numbers. Such disturbances were not serious, and corrected themselves spontaneously. A cholera-like syndrome, however, with severe purging and watery diarrhea, was caused by superinfection with terramycin-resistant staphylococci. This syndrome is not rare, since it was observed in 9 of 200 cases in which terra-

mycin was administered orally. It also was observed in cases in which aureomycin was administered. The appearance of diarrhea with yellow staphylococci in the stools represented an alarming condition and required that administration of the drug be stopped immediately, for the condition could be rapidly fatal. Haight & Finland (88) have reported that treatment with erythromycin was successful in a case in which staphylococcic enteritis followed terramycin therapy for pneumonia. It seems probable that staphylococcic enteritis in patients receiving terramycin or aureomycin will increase with the increasing incidence of aureomycin-terramycin resistant staphylococci. A recent study (89) indicated that 36 per cent of the staphylococci isolated from routine cultures in one clinical laboratory were resistant to aureomycin and terramycin.

NEOMYCIN

Neomycin is a toxic antibiotic which causes deafness as well as renal damage if administered for any length of time. Hamre and her co-workers (90) have studied the activity of several preparations of neomycin. It has been used successfully in the treatment of meningitis and endocarditis due to *Pseudomonas* (91 to 93), as well as in certain resistant infections of the urinary tract (94) and in a case of *Geotrichum* septicemia (95). Neomycin is bactericidal for a wide variety of gram-negative and for some gram-positive bacteria, and is being used in dermatologic practice as a topical application for various pyogenic infections of the skin (96 to 98).

It recently has been used, either alone or in combination with other antibiotics, as an intestinal antiseptic to prepare the intestine for operation (99 to 101). Since it is not absorbed from the gastrointestinal tract to any extent, and since it is rapidly bactericidal, it has some advantages over other agents when used for this purpose. It is not as active against clostridia and *Bacteroides* as the broad-spectrum antibiotics are, but experience indicates that in actual practice the administration of this agent may greatly reduce the number of these organisms.

POLYMYXIN

Polymyxin B has some nephrotoxicity, causes pain at the site of injection, and its use is associated with certain transient neurotoxic symptoms. For these reasons, its systemic administration is employed only when absolutely necessary. It is the most active agent available against *Pseudomonas aeruginosa* (102). Reports of its clinical usefulness for the treatment of infections with this organism continue to appear, including its successful use by the intrathecal route in a case of *Pseudomonas* meningitis (103, 104). It also has been used with success in the treatment of meningitis caused by *Hemophilus influenzae* refractory to other chemotherapeutic agents (105). Brownlee *et al.* (106) have described the pharmacology and chemotherapeutic activity of the several polymyxins, and there have been several studies of the toxicity of the polymyxins (107 to 109).

ERYTHROMYCIN

Erythromycin is a new antibiotic recently described by McGuire and his associates (110). It is derived from *Streptomyces erythreus*. Although it has been available only for a short time, a number of publications involving laboratory and clinical studies make it clear that erythromycin has clinical usefulness and is a welcome addition to the chemotherapeutic agents (88, 111 to 117). Erythromycin is a basic compound that readily forms salts. Its antibacterial activity increases progressively with a rise in pH of the culture medium within the pH range of bacterial growth. It is only moderately soluble in water, but very soluble in alcohol. In general, the antibacterial spectrum is somewhat like that of penicillin. It is more active against streptococci and pneumococci than are the broad-spectrum antibiotics, aureomycin, terramycin, and chloramphenicol. It is active against corynebacteria, *Hemophilus pertussis* and *Micrococcus pyogenes*. It is only moderately active against *Brucella* and *Streptococcus faecalis*. Laboratory studies indicate that it is effective against the rickettsias, large viruses and certain spirochetes.

There is no cross resistance of erythromycin with penicillin, streptomycin, chloramphenicol, aureomycin, terramycin, neomycin, or bacitracin. It is bacteriostatic or bactericidal depending on the sensitivity of the organism and the concentration of the antibiotic. Its *in vivo* activity appears to parallel its *in vitro* activity. Erythromycin is irritating when injected subcutaneously or intramuscularly. It is readily absorbed from the gastrointestinal tract, and, although large doses may cause nausea, vomiting or diarrhea, therapeutically effective amounts can usually be taken without causing these effects. It is readily detectable in the blood and urine, but does not traverse the blood-brain barrier in significant amounts in the absence of inflammation. Organisms may become resistant to erythromycin *in vitro* or *in vivo*, but the process is not as rapid as is usually the case with streptomycin. One of its main uses thus far has been in the treatment of staphylococcic infections resistant to other antibiotics.

CARBOMYCIN

Carbomycin, another new antibiotic produced by *Streptomyces*, has only recently been described by Tanner *et al.* (118). Like erythromycin, carbomycin is mainly active against gram-positive bacteria (117, 119). Animal experiments have demonstrated its ability to protect against infections with certain rickettsias and large viruses. Resistance *in vitro* is acquired gradually. There is no significant cross resistance between carbomycin and penicillin, terramycin, streptomycin, aureomycin, chloramphenicol, polymyxin, or bacitracin. It has been found, however, that with staphylococci and certain strains of streptococci, there is cross resistance between carbomycin and erythromycin (120).

RESISTANCE TO ANTIBIOTICS

Reports which continue to accumulate indicate an alarming increase in resistance of staphylococci to antibiotics. This increased resistance is most

striking with penicillin (121 to 124) but is occurring also with aureomycin and terramycin (125 to 127). There also has been some increase in resistance of the enteric bacteria to aureomycin and chloramphenicol (128, 129). The highest incidence of resistant staphylococci occurs in hospital wards where the most antibiotic treatment is being given, that is, in the surgical wards. In one survey, 51 per cent of a group of operating-room personnel were nasal carriers of staphylococci and 79 per cent of these strains were resistant to penicillin (121). It is evident that cross infection is widespread. With the high incidence of resistant organisms, there is so much strain variation that it is more necessary than ever to carry out sensitivity tests as a guide to therapy.

Starr & Reynolds (130) have called attention to the occurrence of streptomycin resistance of the coliform bacteria in turkeys fed streptomycin in the diet. It may be that the antibiotic feed supplements being given to domestic birds at the present time will create a large reservoir of drug-fast enteric pathogens in the poultry population.

For studies of microbial selection, Bryson & Szybalski (131) have described a gradient-plate technic for the investigation of bacterial resistance and a turbidostatic selector—a device for the automatic isolation of bacterial variants. They used the gradient-plate technic to make strains of *Escherichia coli* resistant to 15 antibiotics (132). Studies of the cross-resistance of these strains led these authors to conclude that the antibiotics could be divided into four major groups.

Matney & Mefferd (133) have described a simple method for selection of organisms resistant to high levels of antibiotics that are sensitive to pH. For instance, with an antibiotic that is most active in an acid environment, a fermentable sugar incorporated into the medium will lower the pH as the organism grows, and will result in the selection of antibiotic-resistant organisms. Gezon & Collins (134) have used an inverted filter candle to hold a culture to be made resistant. This filter candle is suspended in a liquid medium containing the antibiotic. More of the antibiotic can easily be added to the external medium from time to time.

Jensen (135) said that experiments on guinea pigs indicate that tubercle bacilli in the living body acquire a form of streptomycin resistance which differs from that which can be measured by experiments *in vitro*. This form of resistance is brought about when the tubercle bacilli cease to multiply, and assume a resistant, quiescent state. Eagle (136) likewise has suggested that treatment failures with penicillin not only may be due to low concentrations of the drug or to resistant organisms but also to the fact that, in cases of older infections, organisms may no longer be multiplying rapidly and so are insensitive to the effect of penicillin.

Hotchkiss (137) has transferred resistance to penicillin in pneumococci by a method similar to that used for capsular-type transformation. Penicillin-resistant variants occurred when a penicillin-sensitive, rough strain was grown in the presence of desoxyribonucleic acid prepared from rough strains selected for spontaneously arising penicillin resistance.

The mechanism of the development of increased bacterial resistance to antibiotics continues to attract attention, and whether it is by adaptation or by the selection of resistant mutants is the main field of contention. In 1951, Eagle (138) observed progressive increase in the resistance of organisms to an antibiotic when they were placed in threshold concentrations of the agent. This low-level resistance was attributed to progressive adaptation of the total population to the drug. In a later paper, these observations were described in detail (139), but it also was stated that the evidence that highly resistant organisms in bacterial cultures represent spontaneous mutants seems reasonably conclusive. Likewise, Hughes (140) found that with staphylococci exposed to penicillin the number showing increased resistance depended upon the closeness of the steps in penicillin concentration used in testing the resistance.

Sevag & Rosanoff (141) studied the resistance of staphylococci to streptomycin. They found that staphylococci obtained from a medium free of streptomycin do not contain streptomycin-resistant mutants, and that growth in the presence of streptomycin in a complete medium results in the appearance of resistant organisms from sensitive organisms after they have been acted on by streptomycin. Their evidence indicated that resistance was induced, and they criticized the theory of resistance based on the selection of resistant mutants on the grounds that such an effect required the presence of the antibiotic and the process involved might actually be the selection of drug-induced mutants.

This criticism of the theory of the development of resistance by mutation and selection has been controverted by an ingenious device described by the Lederbergs (142), in which, by means of a piece of velveteen stretched over a cylindric piece of wood or cork, a master impression from a culture plate may be used to give replica-inocula to subsequent plates impressed in the same way. Replicas to agar containing streptomycin showed that mutants of *Escherichia coli* resistant to the drug existed in clones on the initial plates of indifferent agar medium. These observations prove that resistance to antibiotics may develop by spontaneous mutation and natural selection.

COMBINATION THERAPY

The use of combination therapy in tuberculosis has been discussed under the heading "Streptomycin." Kirby (143) has comprehensively reviewed the subject of synergism and antagonism with combinations of antibiotics in the previous volume of the *Annual Review of Microbiology*. Combinations in general clinical use are penicillin and streptomycin for subacute bacterial endocarditis or meningitis due to enterococci (144 to 146), streptomycin and aureomycin or streptomycin and terramycin for brucellosis (147), and streptomycin and *p*-aminosalicylic acid for tuberculosis (16, 17). Other combinations have been used with advantage, but it is evident that there are such differences in strains of bacteria, even within the same species, that it is difficult to generalize.

In vitro bactericidal or inhibition tests with different combinations have sometimes served as a guide for the treatment of refractory infections. Rantz & Randall (148) recently have described methods they have used for testing the effect of various combinations of antibiotics on strains of bacteria obtained from patients. Ahern & Kirby (149) successfully treated a patient with subacute bacterial endocarditis caused by streptococci with a combination of penicillin and chloramphenicol after other methods had failed. This combination was used because of the results of *in vitro* tests with the organism. Such a combination is unfavorable *in vitro* with most strains of streptococci since chloramphenicol may interfere with the bactericidal effect of penicillin. Further, it appears that synergistic or additive effects of certain antibiotic combinations demonstrated *in vitro* may not be advantageous in clinical practice. De & Das (150) found chloramphenicol and terramycin to be an advantageous combination against *Salmonella typhosa in vitro*, but no better than chloramphenicol alone in the treatment of patients with typhoid fever. The results of a laboratory study of combinations of antibiotics by Bliss and her co-workers (151) indicate the varying results which may be obtained when the conditions of the experiments are altered, and reflect the difficulty of attempting to apply the results of laboratory studies of combinations of antibiotics to clinical practice.

Reid and his co-workers (152) have reported the results of *in vitro* studies of combinations of different antibiotics against a series of gram-negative rods. Waisbren & Carr (153) found penicillin with chloramphenicol to be an advantageous combination for the treatment of infection with *Proteus*, and Pomerantz (154) successfully treated a patient with refractory *Pseudomonas* septicemia and pyelonephritis with a combination of aureomycin and streptomycin.

By using spirochetes of relapsing fever, Ercoli & Carminati (155) found that the antispirochetal activity of arsenoxide was enhanced by combined treatment with streptomycin, penicillin or bacitracin, whereas terramycin, aureomycin, or chloramphenicol interfered with the action of arsenoxide. Kolmer (156) found a synergistic or additive therapeutic effect of arsenoxide and terramycin in the treatment of experimental syphilis of rabbits.

In view of Jawetz's observation that chloramphenicol interfered with the action of penicillin when mice infected with hemolytic streptococci were treated with a single injection of both antibiotics, experiments to test the value of continuous treatment of a hemolytic-streptococcic infection of mice for several days with combinations of antibiotics were undertaken by two different groups of workers. Speck & Jawetz (157) found an antagonism to the action of penicillin by either chloramphenicol or terramycin, whereas the results of the study by Ahern, Burnell & Kirby (158) indicated that chloramphenicol plus penicillin protected the mice as well as did penicillin alone, and that the phenomenon of antibiotic interference was seldom of clinical significance.

An *in vitro* study of antibiotic synergism and antagonism has been

reported by Jawetz *et al.* (159). It was stated in their paper that, "It remains to be established whether the type of synergistic effect described here bears any relation to the desired therapeutic action of combinations of antibiotics in vivo."

ANTIBIOTICS IN PARASITOLOGY

Evidence continues to accumulate that the antibiotics aureomycin, terramycin, neomycin, and bacitracin are of value in the treatment of amebiasis (160 to 164). Relapses are frequent, however, and there is a report of an acute attack of amebic dysentery which developed during a course of treatment with aureomycin for a pre-existing condition (165). Attempts to develop resistance of *Endamoeba histolytica* to aureomycin or terramycin during thirty-eight serial transfers in medium containing partially effective concentrations of the agents failed (166). Two reports indicate that fumagillin is an effective antiamebic substance for the treatment of infections of human beings, although with large doses there are untoward effects (167, 168). In experimental infections with *E. histolytica* in guinea pigs, fumagillin was the only antibiotic found therapeutically active (169). Thiolutin and rimocidin, recently described antibiotics (170, 171), have been found to be active *in vitro* against *E. histolytica* and certain leishmanias and trypanosomes. Achromycin, an antibiotic obtained from a new species, *Streptomyces albo-niger*, has a protective effect in mice infected with *Trypanosoma equiperdum* (172). Vaginitis caused by *Trichomonas vaginalis* has been treated by the local application of terramycin or aureomycin with somewhat variable results (173, 174).

Wells (175, 176) found that terramycin, aureomycin, and bacitracin caused a reduction in the worm burden in infections with the mouse pinworm, whereas administration of neomycin, dihydrostreptomycin, and chloramphenicol resulted in an increase in the worm burden. Anaplasmosis carrier infection in cattle was destroyed by treatment with aureomycin (177), and experimental Nosema disease of honeybees has been partially controlled by feeding fumagillin (178). It is evident that there is considerable interest in antibiotics in the field of parasitology.

EFFECT OF ANTIBIOTICS ON FUNGI

There has been a continuing interest in the search for antibiotics that will affect the fungi. Ascosin, an antibiotic from a new species of *Streptomyces* (179), is active against yeasts generally and against some filamentous fungi. It is evident from the preliminary studies that it has some animal toxicity. Thiolutin has considerable activity against a number of fungi causing both systemic and superficial infections (170), and has been used with some success in the treatment of tinea capitis (180). Several other antibiotics active against fungi have been described; among them are helixin (181) and an agent of the fungicidin type (182). Florestano & Bahler (183) have called attention to the fact that polymyxin has some fungistatic action. Actidione

has been used in greenhouse experiments to control covered smut of wheat by rapid treatment of the seed (184).

EFFECT OF ANTIBIOTICS ON VIRUSES

There is little that is encouraging or new in the field of therapy of infections caused by viruses. No effect was found when aureomycin, terramycin, or chloramphenicol was administered to patients with mumps (185 to 187) or when aureomycin or chloramphenicol was given in cases of herpes zoster (188, 189).

Finland (190) has reviewed the reports of the treatment of primary atypical pneumonia with aureomycin, and concluded that it seems justifiable to consider that aureomycin is an effective agent in primary atypical pneumonia until more adequate and more thoroughly controlled data are obtained. Finkel & Sullivan (191) found aureomycin to be of therapeutic value in treating patients acutely ill with primary atypical pneumonia. A study of the cold-agglutinin titers in cases of primary atypical pneumonia (192) indicated that in cases in which aureomycin was not administered the titers of cold agglutinins were higher and were sustained for a longer time than they were in cases in which this antibiotic was administered.

The course of acute viral hepatitis was not significantly changed by treatment with aureomycin or chloramphenicol (193).

It is stated by Krishna Murty (194) that, in the treatment of trachoma, the sulfonamide drugs, as well as penicillin and streptomycin, are able to affect only the secondary infections, whereas aureomycin and terramycin affect not only the secondary infective organisms but also the trachoma virus and inclusion bodies. According to Mitsui (195), the inclusion bodies of acute trachoma respond promptly to local treatment with terramycin or aureomycin, and these drugs bring about a radical cure without exception. This enthusiasm is not shared by everyone, however. Cat (196 to 198) stated that aureomycin or terramycin applied locally following curettage is a valuable agent for the treatment of trachoma: these drugs have a dramatic effect on secondary infections and minor complications, but they cannot be considered as medicaments specific for trachoma. Likewise, Siniscal (199) has found that the antibiotics, including penicillin, bacitracin, streptomycin, chloramphenicol, aureomycin, and terramycin, have no effect on trachoma per se, but that they are excellent for treating secondary infections associated with trachoma. He expressed the opinion that the sulfonamide drugs are superior to all other therapeutic agents used thus far for this disease.

A study of experimental vaccinia-virus infection in the chick embryo and in the rabbit treated with aureomycin showed that this agent does not have any effect on the development of the virus (200). Neither aureomycin nor terramycin had an effect on the course of rabies in white mice when street virus was inoculated into the masseter muscle (201).

Groupé et al. (202) have found that a material produced by a culture of Achromobacter was capable of suppressing the development of pulmonary

lesions in mice previously infected with influenza A virus, but that it failed to affect the production of infectious virus in lung tissue. Powell and his associates (203) found a fungous filtrate (*Penicillium* strain) which has chemoprophylactic action in mice against MM and Semlike Forest virus when filtrate and virus are given by separate routes.

Asheshov and co-workers (204) have described an antibiotic named "phagolessin A58," which has a strong phagocidal action against a large number of bacterial viruses.

ANTIBIOTICS IN THE TREATMENT OF THE RADIATION SYNDROME

Furth and associates (205) treated dogs with aureomycin following irradiation of the whole body with roentgen rays. Bacteriologic studies showed a higher percentage of positive blood cultures in the control dogs than in the treated dogs. Gross findings at necropsy did not reveal any gastrointestinal ulceration but disclosed minimal evidence of hemorrhage in the treated group while marked gastrointestinal ulceration and hemorrhage were found in the control dogs. The mortality rates in the two groups were not significantly different, but in the treated group death occurred later than it did in the control group.

In experiments on rats by the same workers (206), terramycin delayed the onset of death, and reduced the mortality following total irradiation of the body. Miller and his co-workers (207) subjected mice to irradiation of the entire body and treated them with a variety of antibiotics, generally from the fourth to the twenty-eighth day after irradiation. The administration of streptomycin resulted in the greatest reduction in the mortality rate. Chloramphenicol, aureomycin, and terramycin all caused significant reductions in mortality. Neomycin was ineffective, and polymyxin was too toxic.

It is evident that infection is one of the contributing factors to the mortality of animals following experimental irradiation with roentgen rays, and that antibiotics can be used to control this complication.

ANTIBIOTICS IN GROWTH AND NUTRITION

The discovery that the addition of a small amount of certain antibiotics to the diet favorably influenced the growth and nutrition of young animals of a number of species opened up new fields for investigation and commercial activity. The literature contains many articles on the effect of antibiotics on animal growth and nutrition. Large amounts of antibiotics are now produced solely for addition to animal diets. Since this field has not been considered in previous editions of the *Annual Reviews of Microbiology*, some of the original papers will be mentioned.

In 1946, Moore and his co-workers (208) reported that the addition of succinylsulfathiazole or streptomycin to a purified diet led to increased growth in chicks. Stokstad and his associates (209) observed that a fermentation product of *Streptomyces aureofaciens* promoted growth in chicks.

This favorable effect was later found to be due to the aureomycin which was present (210, 211). Lesser responses were also produced with succinyl-sulfathiazole, streptomycin, and 3-nitro-4-hydroxyphenyl arsonic acid. Other workers also had described the stimulation of growth of chickens and turkeys by derivatives of phenylarsonic acid (212 to 215).

That the observations made on chicks might have a general application to animal nutrition was indicated when Jukes and his associates (216) found that crystalline aureomycin added to a diet with vitamin B_{12} resulted in extra growth of pigs. A similar result was obtained with streptomycin in pigs by Luecke and his co-workers (217).

Antibiotics as metabolites.—Studies with the chicken by one group of investigators (218) indicated that the parenteral administration of antibiotics and of autoclaved penicillin increased the rate of growth but had little effect on the fecal aerobic microflora. It was surmised that the antibiotic molecule or a fragment of the same might act as a metabolite within the body of the bird.

The results of studies by other authors (211; 219 to 221), however, with both chicks and rats, are contrary to these findings and indicate that parenterally injected antibiotics and the feeding of inactivated antibiotics do not have any stimulating effect on growth.

Stimulation of growth by increasing feed consumption.—Experiments performed by Scott and Glista (222) by using highly fortified diets indicated that aureomycin caused a slight early stimulation of growth by increasing feed consumption. The data of Davis & Briggs (223), however, showed that increased growth of chicks which received diets containing aureomycin, penicillin, bacitracin, terramycin, or streptomycin was due to improved feed efficiency and was not caused by increased feed consumption.

Relative growth-stimulating effect of different antibiotics.—The comparative effect of several antibiotics on the growth of young turkeys was determined by McGinnis and his co-workers (224). Aureomycin, terramycin, streptomycin, and penicillin, as well as 3-nitro-4-hydroxybenzene arsonic acid, all caused an increased gain in weight when added to the diet. Penicillin was the most effective in this respect. The addition of 5 mg. of this antibiotic to each kilogram of the diet caused a greater growth than did the addition of larger amounts of other antibiotics. Combinations of other antibiotics with penicillin did not cause greater growth than did penicillin alone.

That the relative activity of different antibiotics just mentioned may not hold true for different species of animals is suggested by the fact that White-hill and his associates (211) found no difference in the growth of pigs given aureomycin, streptomycin, or penicillin while receiving a corn-soybean meal basal diet. Cunha and his co-workers (225) reported, however, that penicillin did not stimulate growth in pigs, that streptomycin had some effect, and that aureomycin was most active in this respect. These antibiotics were used with a corn and peanut-meal basal diet. It may be that the diet used, as well as the species of animal, may determine the value of an individual antibiotic for stimulating growth.

A recent study by Becker and his co-workers (226) indicated that aureomycin and terramycin were the most active of several antibiotics for stimulating the rate of gain in swine, and that 5 mg. of the antibiotic per pound of diet was effective. In experiments with turkey poults (227), it was found that there was a difference in two varieties in the response to antibiotics when given in the same concentration.

Carbomycin, another antibiotic which recently has been described (118), has been found capable of stimulating the growth of chicks when incorporated into an all-vegetable ration containing vitamin B_{12}.

Sparing effect on vitamins.—The most favored concept for the cause of the growth-stimulating effect of adding antibioitcs to the diet of young poultry and swine is that it is due to changes produced in the bacterial intestinal flora (228 to 234). The results of a number of studies indicate that the growth-promoting action of antibiotics may be associated with a sparing effect on certain elements of the vitamin B complex which are present in the diet in limited amounts. It has been postulated that an antibiotic may conserve a given vitamin for the host by inhibiting the growth of organisms in the intestinal tract that would compete with the host for that vitamin. March & Biely (232) found that lactobacilli were the first of the fecal bacteria in chicks to be affected by feeding aureomycin. The lactic acid bacteria are known to have specific requirements for many of the vitamins, and it is possible that these bacteria are competing with the chick for some of the vitamins. Thus the suppression of the lactic acid bacteria may be a factor in the growth-stimulating effect of aureomycin for the chick.

That antibiotics in the diet exert a sparing action on vitamin B_{12} has been shown by experiments on poultry, rats, and swine (221; 235 to 238). Johansson and his associates (234) found that feeding aureomycin stimulated the growth of baby rats which received purified rations limited in methionine or vitamin B_{12} or both, but that it was without effect on the growth of rats fed a practical diet. Vitamin B_{12} was synthesized in the intestines of rats fed rations (with or without aureomycin) deficient in this factor. No conclusive effect of aureomycin on the concentration of vitamin B_{12} in the intestinal tract was demonstrated.

It has become evident that B_{12} is not the only vitamin concerned in stimulation of growth by antibiotics. Sauberlich (239) designed an experiment to determine if antibiotics would have a sparing effect upon the vitamin requirements of the rat. It was found that penicillin added to the diet (0.01 per cent) resulted in a marked stimulation of growth in rats fed diets free of or low in thiamine, pyridoxine, pantothenic acid and, to some extent, in riboflavin. Aureomycin (0.01 per cent) had a similar effect in the case of deficiencies of pantothenic acid and thiamine. The addition of penicillin or aureomycin to a completely vitamin-supplemented basal diet had no effect on growth.

Linkswiler and his associates (240) found that aureomycin increased the growth of rats fed a diet limited in pyridoxine. Lih & Baumann (241) reported

that penicillin, aureomycin, and streptomycin stimulated the growth of rats receiving limiting amounts of thiamine, riboflavin, or pantothenic acid. In thiamine deficiency, penicillin produced the greatest increases in growth. In riboflavin deficiency, penicillin and aureomycin were equally effective. In pantothenic acid deficiency, aureomycin and streptomycin were best. Waibel *et al.* (242) found that the addition of penicillin in amounts of 5 and 200 mg. per kilogram to a practical breeder mash resulted in deposition of an increased amount of biotin and folic acid in the egg yolk.

In contrast to these findings, it has been reported (243, 244) that the oral administration of large amounts of certain antibiotics may result in vitamin deficiency in man. The role played by the flora of the intestine in the synthesis and utilization of vitamins in the human being has not been determined. It may be, however, that the oral administration of broad-spectrum antibiotics eliminates a bacterial flora which normally supplies vitamins valuable for human nutrition. The elimination of most of the intestinal bacteria results in marked growth of yeast in the feces (245). It has been found that feeding live yeast to human beings caused thiamine to become unavailable for absorption (246), and it seems possible that the growth of large numbers of yeasts in the intestine may contribute to a thiamine deficiency.

Sarett (247) has studied the effect of oral administration of streptomycin on the excretion of vitamin B complex in the urine of man. Administration of the antibiotic resulted in complete or partial inhibition of growth of coliform organisms and almost complete absence of the other aerobic organisms studied. Excretion of biotin, was markedly decreased during administration of streptomycin. This suggests that the intestinal flora may furnish some biotin for nutrition in man.

Relation of dietary protein and carbohydrate to the growth-response to antibiotics.—A number of workers have concluded that antibiotics do not have a sparing action on the dietary protein or carbohydrate requirements of the chick (248 to 253) Heuser & Norris (254) found that a greater relative growth response with antibiotics was obtained in chicks fed vegetable-protein rations than in those fed rations containing animal protein.

Studies with the laboratory mouse by Vijayaraghavan and his co-workers (255) indicate that this is another animal in which the growth may be stimulated by an antibiotic. Aureomycin added to a natural food ration, purified casein diets and to a peanut-meal diet did not produce any significant change in growth. Marked stimulation of growth, however, occurred when the antibiotic was added to diets based on soybean meal and cottonseed meal. The authors suggested that the growth stimulation may result from promotion of the development of flora which enhance the availability or the intestinal synthesis of critical amino acids. When diets containing proteins of high value for nutrition are provided, these amino acids are not limiting factors for growth, and aureomycin added to the diet does not cause any stimulation of growth.

There is also evidence that the nature of the dietary carbohydrate can affect the growth response to antibiotics (256).

Is the growth-stimulating effect of antibiotics due to suppression of toxin-forming clostridia?—The finding that penicillin and terramycin inhibited the growth of *Clostridium perfringens* in the ceca of turkeys led Sieburth *et al.* (228) to suggest that stimulation of growth was a result of the elimination of these toxin-producing organisms. Williams and his associates (229), however, have recently shown that oral or cloacal administration of combined sterile toxins of *Cl. perfringens, Cl. septicum, Cl. histolyticum, Cl. tetani, Cl. novyi,* and *Cl. oedematoides* did not have any effect on the growth of chickens, nor did administration of antitoxins for these organisms.

Smyser and his co-workers (257) found that there were only slight changes in the number of *Cl. perfringens* over a six-week period in chickens receiving aureomycin or penicillin in the diet. With penicillin, there was an actual rise in number by the fourth week. Larson & Carpenter (258) found that pigs receiving penicillin, aureomycin, or chloramphenicol in the diet had fewer *Cl. perfringens* in the feces than did pigs which received antibiotic-free rations, but that there was no correlation with growth.

These findings fail to support the hypothesis that clostridia in the feces elaborate toxins which inhibit growth and that the growth-stimulating effect of antibiotics in the diet is due to suppression of these clostridia.

Stimulation of growth by suppression of infection.—That antibiotics may favor growth in poultry by suppressing an easily transmissible infection is suggested by studies cited in a recent review article (259). Chicks gained weight normally in an environment where chickens had not previously been housed, but with chicks which were obtained from the same source and fed the same diet quartered where chickens had been kept before it was necessary to add penicillin to the diet to obtain a normal rate of growth. Todd and Todd & Stone (260, 261) found that penicillin inhibited the development of *Ascaridia galli* in the intestines of chickens.

It is evident that the mode of action of antibiotics in stimulating growth in animals is not understood. It is most generally believed that the effect results from their influence on the intestinal flora. The reduction of organisms competing for micronutrients, the inhibition of toxin-producing organisms, or a selective inhibition favoring organisms producing certain vitamins is a possible explanation for the effect. It also has been suggested that antibiotics may favor the development of a flora which increases the availability, or the intestinal synthesis, of important amino acids.

In some experiments, the evidence suggests that the antibiotic favors growth by eliminating an infection that is readily transmissible, and endemic to a certain locality, or that a widely distributed intestinal parasite is inhibited in animals receiving the antibiotic. Certain studies tend to show that the addition of antibiotics to the diet stimulates growth by increasing feed consumption. It has also been postulated that the antibiotic molecule, or a fragment of it, is utilized as a metabolite by the host.

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MEDICAL MYCOLOGY[1]

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Since this is the first time that this topic has been treated in these Reviews, it is manifestly impossible to cover the broad subject of the title without some arbitrary limitation on the period of time to be covered. Fortunately, symposia volumes (1, 2) covering much of the literature through 1947 permit consideration to be confined in most instances to papers published from 1948 through 1952. However, work of exclusively clinical interest has not been surveyed, and the subject of the immunology of fungus infections, which was reviewed (3) in 1950, will be omitted from consideration.

The person entering into research in medical mycology, or seeking an introduction to the subject, now can find a number of excellent texts and reviews. This was hardly the case before the last war and is an indication of the rapidly mounting interest and outpouring of research on medical mycology. *The Manual of Clinical Mycology* by Conant and co-workers (4) set a new standard almost at the beginning of this surge of activity and has been of great importance to all engaged in the subject. New editions of the standard texts by Lewis & Hopper (5), and Swartz (6), have appeared and the *Précis de Mycologie* of Langeron, which has been revised and enlarged by Vanbreuseghem (7), devotes much attention specifically to medical mycology. Ainsworth (8) has published a small volume that comprises a series of stimulating essays on topics of general biological interest in medical mycology. Among the more recent monographs, Mackinnon's volume (9) supplies a detailed account of pathogenic yeasts; in this same field an excellent volume on *Cryptococcus neoformans* has been published by Cox & Tolhurst (10), while Skinner (11) has reviewed the genus *Candida* in authoritative fashion. It would not do to leave the matter of works of general interest without mentioning the medical mycology abstracts, published semiannually as the *Review of Medical and Veterinary Mycology* (a continuation of *An Annotated Bibliography of Medical Mycology*, 1943 to 1951) under the editorship of Wiltshire (12).

Although it is difficult to treat problems of taxonomy in the short compass of a review, it is realized that a very real barrier to progress in medical mycology has, in the past, been the cumbersome burden of trivial binomials created at the least suspicion of variation from the supposed standard. Through the efforts of Emmons, Conant, Benham, Langeron, Ainsworth, and others a reasonable nomenclatural structure is being evolved that is in

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[1] The survey of literature pertaining to this review was concluded in December, 1952.

working agreement with clinical, physiological, and ecological studies. Two lists of recommended names of pathogenic fungi have appeared recently: a memorandum of the Medical Research Council of Great Britain (13), and a report of the Committee on Medical and Veterinary Mycopathology of the International Association of Microbiologists (14). It would be unfortunate were a list of recommended names to become a block to the acceptance of genuine taxonomic advances, but such a situation would be something of a novelty in medical mycology which has seen the plethora of names for frequently identical organisms make studies on comparative physiology and geographical distribution, at the least, difficult.

MYCOTIC INFECTIONS AND PUBLIC HEALTH

The increasing importance from the public health standpoint of mycotic infections in the United States has been emphasized by Salvin (15), Ajello (16), Gordon (17), Christie (18), and Lee (19). Aspects of the problem in Canada have been outlined by Fischer & Wrong (20), in Brazil by Almeida, Lacaz, and Costa (21), in Denmark by Sylvest (22), in England, by Duncan *et al.* (23, 24) and Ainsworth (25) and by Mackinnon (26) in Uruguay. The list might be expanded greatly, but the trend is evident from these selections. Rather than being a remote problem in medicine and public health, mycotic infections are probably the most widely distributed and most numerous types of infections. Dermatophyte infections are probably as prevalent as the most widespread of the bacterial or virus diseases. Figure 1 shows the relative importance of mycoses among the causes of death resulting from infectious and parasitic diseases. These data, obtained from the Vital Statistics Reports (27, 28), show that in the United States mycoses accounted for 0.56 per cent of all deaths from infectious disease in 1949 (latest reports available) and now stand 14th in point of frequency. The number of deaths from mycoses in 1949 exceeds the total of all deaths attributed to infections by protozoa, rickettsiae, and helminths. The system of listing cause of death in the Vital Statistics Reports was altered, beginning with data for 1949, and comparative tabulations as shown in Table I illustrate the magnitude of serious mycoses. If the trends shown in Figure 1 are not shortly reversed, mycotic infections will assume even greater importance. The graph is a thumbnail sketch of the "age of antibiotics," but it is evident that the antibiotics referred to are antibacterial, and not antifungal.

GEOGRAPHICAL DISTRIBUTION OF MYCOTIC INFECTIONS

Since the review by Martin (29) in 1947, much new information has become available on this subject. There is need for a thorough monograph which will bring together the tremendous literature which has appeared in the last few years on the distribution of mycoses, skin sensitivities, soil isolations, and animal reservoirs. Correlation of these data with climatological data, in view of the implication of dust storms in the potentiation of
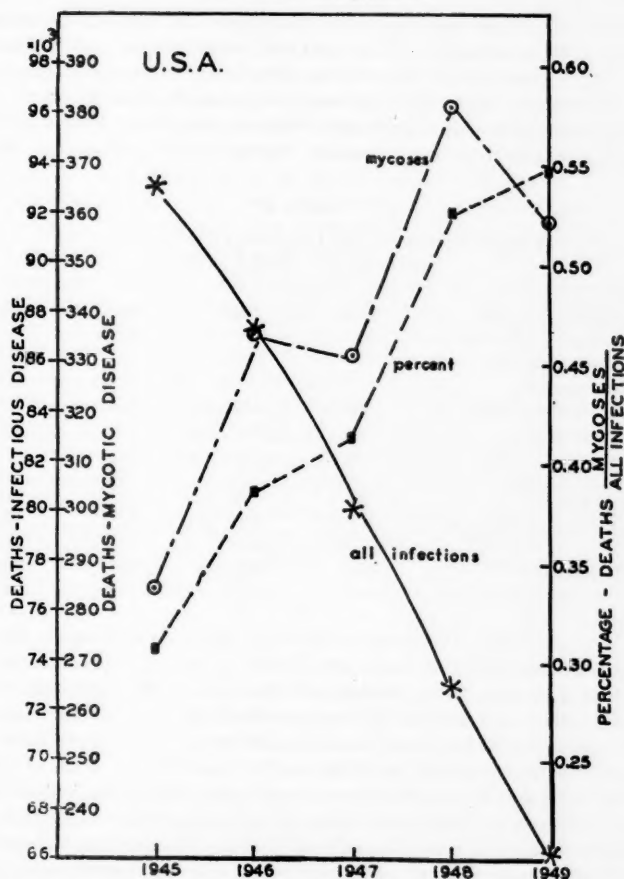
FIG. 1. Deaths in United States from fungus infections, 1945 to 1949, compared with deaths in similar period from all infectious diseases. Data from Vital Statistics (27, 28); data for 1949 corrected for new classifications to put total infectious disease deaths on a comparable basis with that of 1945–48.

certain pulmonary mycoses (30), and with the incidence of nontubercular pulmonary calcification, in view of the important findings of Smith (31) and Christie (18), may well lead to a complete revision of our thinking on the prevalence and importance of mycotic infections.

Coccidioidomycosis.—Although the original isolation of *Coccidioides*

immitis from an infection caused by this fungus was made in Argentina by Posada in 1891, it is only recently that additional infections with *C. immitis* have been reported from that country (32). Despite this, the Negronis (33) find that slightly more than 8 per cent of a population sample reacted positively in skin tests with coccidioidin. Tests on more than 2000 children revealed more than 10 per cent of positive reactors to be in one district, an arid,

TABLE I

MORTALITY FROM FUNGUS INFECTIONS IN UNITED STATES
FOR YEARS 1946–1949 (27, 28)

Cause of Death	1946	1947	1948	1949
Actinomycosis	—	—	—	32
Blastomycosis	—	—	—	27
Coccidioidomycosis	46	42	63	83
Cryptococcosis	—	—	—	35
Dermatophytosis	—	—	—	4
Histoplasmosis	—	—	—	33
Moniliasis	38	49	57	94
Other fungus infection	252	240	261	52
All Mycoses	336	331	381	360

dusty, steppe region. Prevailing winds focus onto this arid region and may increase the likelihood of pulmonary injury by dust and promote the distribution of fungus spores. Irrigation of the soil has been proposed to control the endemic soil fungus (34). An extensive study of coccidioidin sensitivity in other South American countries (35) revealed an appreciable incidence of positive reactions in arid zones. It is possible that areas of endemism in arid regions are essentially continuous either side of the Andean chain. A clue to the existence of links between the foci of the disease in South America and the intense concentration of coccidioidomycosis in the arid Southwest of the United States is given by the first report of a case from Central America. Castro & Trejos (36) report the isolation of *C. immitis* from a person long resident in the Comayagua Valley of Honduras. In this area, 4.1 per cent reactors to coccidioidin were observed in the hospital of Lima (37), whereas only 1 per cent reactors were found at Tela on the north coast of Honduras (38).

The interrelationships among animal reservoirs (39), soil reservoirs (40), dust storms (30), and incidence of *C. immitis* infection (41) are in large measure to be worked out. Fortunately, however, the striking similarities among many of the features of the pulmonary mycoses are currently causing an interfertilization of thought on the problems common to the study of these

infections, as witness studies on the correlation between antigen sensitivity and pulmonary calcification (18, 31, 41, 42, 43). These studies are, in a sense, impelled in view of the high incidence of coccidioidin sensitivity in certain areas of California and the striking calculation by Ajello (16) that approximately one million people have been infected by *C. immitis* in California alone.

Histoplasmosis.—Paralleling the history of the isolations of *C. immitis* in Argentina, the first isolations of *Histoplasma capsulatum* by Darling in the Pamama Canal Zone in 1906–07 were not succeeded by other reports until 1951, when Draheim, Mitchell, & Elton (44) reported the isolation of the fungus from an infant. In the intervening years a considerable literature has accumulated on this disease. One of the most interesting developments grew out of a suggestion by Smith (31), based on his studies on coccidioidomycosis, that pulmonary calcification in tuberculin-negative, coccidioidin-negative foci in the lower Mississippi River region could originate from *H. capsulatum* infections. The data of Christie & Peterson (45), of Palmer (46), and of Emmons, Olson, & Eldridge (47) constitute very strong evidence that benign forms of histoplasmosis are, indeed, responsible in certain areas for a considerable part of the hitherto unexplained calcification. Histoplasmin sensitivity has been shown to exist in 60 to 84 per cent of large population samples of residents of Mississippi, Tennessee, Missouri, and Kentucky. In these same areas a high incidence of pulmonary calcification was also found, ranging from 56 to 64 per cent of the population sampled. Further studies by Christie (48), Palmer (49), Furcolow (50, 51), and Loosli et al. (52, 53) have borne out the existence of a Central States area wherein histoplasmin sensitivity occurs in over 65 per cent of the large population sample with a correspondingly high proportion of the population having pulmonary calcification. Christie (18, 54) has presented excellent reviews of this work. He has advanced the thesis that infection with *H. capsulatum*, like infection with *C. immitis* or with the tubercle bacillus, can result in overwhelming infection with dissemination and fatal outcome. Depending, however, upon the resistance of the host, the virulence of the organisms, or other unknown factors, the organisms may be rendered nonviable or may be phagocytized. Giant cell formation around the liberated lipid substance may be followed by caseous necrosis with healing and calcification.

The long interval between Darling's discovery of the first cases of histoplasmosis and the next case of the disease in the Panama Canal Zone is of interest in light of the high incidence of histoplasmin sensitivity in that region. Tucker et al., (55, 56) reported a prevalence of 40 per cent reactors in the Canal Zone. Moreover, the organism had been identified in an autochthonous canine infection in 1945 (57).

Emmons (58 to 61) has reported numerous isolations of *H. capsulatum* from dogs, rats, cats, and the spotted skunk, but no isolations from field mice and only one isolation from more than 1000 house mice examined (62).

In contrast, *C. immitis* has been isolated thus far (39, 40) from only three species of desert rodents native to an endemic focus of coccidioidomycosis in Arizona. There may be no causal relationship between the ecological behavior of the respective animal reservoirs of *C. immitis* and *H. capsulatum*, but the findings are in agreement at least with the ecological aspects of the respective diseases in man, the former confined to arid regions, the latter prevalent in river lowlands. Macroconidia of *H. capsulatum* have been found by Emmons (63) in soil samples from Virginia and by Gordon *et al.* (64) in soil and river water in Williamson County, Tennessee, an area with a very high prevalence of sensitivity to histoplasmin. The samples from Virginia were collected on a farm where 7 of 43 rats trapped had histoplasmosis. The soil samples were taken at the entrance to rat burrows.

One other aspect of histoplasmosis may be mentioned. In reviewing a series of 88 cases of histoplasmosis, Cawley & Curtis (65) found four in which lymphoblastoma also occurred. Murray & Brandt (66) have also studied this association and find two more cases in which it has occurred. They comment that the association of diseases may be explicable on the basis of a cytologic reaction of the reticulo-endothelial system to stimulus by a specific infection. Hulse (67) and Hutchison (68) studying laryngeal histoplasmosis pointed to its simulation of carcinoma, and sarcoid-like lesions in cases of fatal, disseminated histoplasmosis have been described by Pinkerton & Iverson (69) and by Israel and associates (70). Histologic features usually associated with Hodgkin's disease were reported by Furcolow (71) in a case of histoplasmosis, and Rodger, Terry, and Binford (72) have described a case of Hodgkin's disease which was complicated by histoplasmosis.

Aspergillus and Aspergillosis.—Ainsworth & Rewell (73) studied 78 cases of aspergillosis in captive wild birds in England. From 68 of these cases there were isolated in 45 instances pure cultures of *Aspergillus fumigatus*, three of *A. flavus*, and one of *A. nidulans*. No differences were observed in the disease produced by these fungi. The thesis that avian aspergillosis is characteristically a disease of captivity is not supported by this study. The disease was found to be especially pronounced in recently-captured water birds. These observations support the view (74) that aspergillosis of birds is endemic in the wild and may become more pronounced under the unnatural conditions of captivity. Avian tuberculosis, on the other hand, is almost certainly confined to captive or domesticated birds. Six cases of mixed infection of fungus and acid fast bacterium were observed in this series and serve to emphasize the generalization that in birds caseous lesions of the lung are almost always mycotic, whereas those of liver and spleen are usually tuberculous. In only one instance, however, was the avian strain of *Mycobacterium tuberculosis* obtained in culture from a mixed infection; the fungus isolated in this case was *Candida albicans*. This observation is interesting in light of the well known antibacterial action of metabolic products of *A. fumigatus. A. fumi-*

gatus has also been isolated in pure culture from a massive overgrowth of the air-passages of a bison (75).

Dermatophytes and Dermatophytosis.—It is impossible to do justice to the vast literature on this subject, and all that will be attempted will be the selection of a few papers of possibly general interest. The frequency of isolation of different species of dermatophytes has been compared by Sagher (76) on the basis of reports from nine laboratories in different parts of the world. Vanbreuseghem (77) has added data based on numerous isolations of dermatophytes in Belgian Congo from whence he describes several new species. Thomas, Lennox & Duncan (24) have surveyed the incidence of various ringworm infections in England, and Vilanova & Casanovas (78) have described an epidemic of trichophytosis in Spain that was confined mainly to persons handling rabbits infected with a variety of *Trichophyton mentagrophytes*.

Tinea capitis after puberty has been found by Pipkin (79) to be more common in southwestern states than heretofore suspected. *T. tonsurans* was the principal organism isolated from a series of 33 adults, in many of whom the fungus infection mimicked other types of scalp lesions. This finding is of some interest in view of the fact that in adjacent Mexico ringworm of the scalp is caused almost exclusively by the endothrix *T. tonsurans*, whereas *Microsporum audouini* infections are not known to occur there, according to Gonzales Ochoa & Vazquez (80). Georg (81) has uncovered evidence indicating that *T. tonsurans* infection is spreading into southwestern states from an endemic focus in Mexico and is assuming the proportions of a new public health problem. The spontaneous cure of *M. audouini* infections at puberty appears not to be paralleled in *T. tonsurans* infections which are known to spread to all members of a family (82).

ISOLATION OF PATHOGENIC FUNGI FROM SOIL

The increasing frequency with which causative agents of systemic mycoses are isolated from soil has directed attention forcibly to the soil as an important reservoir of these infectious agents (83). Clinical and epidemiological observations have emphasized that neither person to person transmission nor direct transmission from an animal reservoir appear to be factors in the spread of systemic mycoses. Many of these infections are sporadic in appearance and are widely dispersed geographically. Frequently a history of injury by a thorn puncture, or with a wood fragment is associated with nonpulmonary systemic mycoses (including sporotrichosis, chromoblastomycosis, blastomycosis, and mycetomas) thus implicating spore introduction accompanying trauma as a major factor in the spread of these diseases. Introduction of air borne spores of fungi causing pulmonary infection simultaneously with inhalation of irritating dust particles may be another pattern of infection following injury.

In Table II is a listing of the isolations of pathogenic fungi from soil or other sources in nature. One obtains a clear impression of the reality of

a natural reservoir, in ecological association with animal reservoirs (61, 83), for infectious agents.

The isolation of *C. neoformans* from soil has been reported by Emmons (86). A soil-flotation, mouse-inoculation method was used. In this technique, essentially a modification of the Stewart-Meyer method (85), a portion of the soil sample is suspended in 100 ml. of physiological saline in a cylinder,

TABLE II

PATHOGENIC FUNGI ISOLATED FROM SOIL OR OTHER NATURAL SOURCE

Organism	Source of isolation
Allescheria boydii	soil (84)
Aspergillus fumigatus	very common in soil
Coccidioides immitis	soil (39, 85)
Cryptococcus neoformans	soil (83, 85)
Histoplasma capsulatum	soil (64, 83, 87, 88), river water (64)
Microsporum gypseum	soil and river water (64)
Nocardia asteroides	soil (89)
Phialophora verrucosa (*Cadophora americana*)	wood (90)
Sporotrichum schenckii	plants, mine timbers (91), florists sphagnum (83), soil (83)

stirred vigorously with a sterile wooden applicator, and allowed to settle at room temperature. After 2 to 3 hr. a 5 to 10 ml. sample of the suspension is pipetted from the upper portion of the liquid and 1 ml. is injected into each of four to eight mice. The mice are killed 4 to 6 weeks after injection and cultures are made, on a Sabouraud-type medium, from the spleen and liver. The strains isolated from soil showed some variation from each other, just as have strains isolated from infections in man, but all of the strains isolated produced acid slowly in glucose and in sucrose and were lethal for mice on intracerebral inoculation.

Ajello (84) reported the isolation of four strains of *Allescheria boydii* (perfect stage of *Monosporium apiospermum* and a causative agent of mycetomas or Madura foot) from 194 samples of soil collected in Williamson County, Tennessee. Three of these strains readily formed perithecia, and a spore suspension from one, when mixed with gastric mucin, was pathogenic for mice. In this paper Ajello points out some of the many pitfalls which may invalidate, or bring into question, a report of the isolation of a suppposed pathogen from nature. A listing of the proved cases of infection caused by *A. boydii* typifies the sporadic distribution of many of the systemic mycoses. Of 25 cases from which this fungus had been isolated up to 1951, 8 had been reported from the United States, 5 from Italy, 8 from South Ameri-

ca and the Caribbean area, and 4 from French North Africa. In the majority of these cases a history of foot injury was reported.

Biological control of soil borne fungus pathogens.—Soils in which *H. capsulatum* has been found to occur are frequently in protected sites, are acid in character and of slow permeability (93). Correlation of soil types with prevalence of the fungus may shed light on the geographic distribution of histoplasmin sensitivity. Although the distribution of pathogenic fungi in soil may explain some of the anomalous aspects of geographical distribution of certain fungus diseases, it is also necessary to consider the persistence of fungi in soil types from which they are not normally isolated. The concept, developed by Waksman, of antibiosis among members of the microbial population in a soil may account, in part, for the failure to recover *H. capsulatum* in a given soil type although the organism may be relatively abundant in a neighboring soil of different type. Recently, there have been many studies on the stability of antibiotics in soil (95 to 98). Jeffreys (95) has distinguished four types of antibiotic inactivation in soils: acidity, biological inactivation, adsorption of basic molecules, and some type of chemical inactivation. Of 10 antibiotics studied, 8 were products of fungi isolated from the soils of the region and in which production of antibiotics under natural conditions has been suspected (99, 100). The rate of inactivation of the different antibiotics varied from soil to soil, but all showed a fair degree of stability in some of the soils.

It is well known that an acidic, slowly permeable soil will probably harbor very few actinomycetes (101), but it is likely that other methods for the suppression of pathogenic fungi in soil may well be more important than control by metabolic products of actinomycetes or by other fungi. The acid soils from which *H. capsulatum* has been isolated (93) are certainly types with a much smaller biota than would be presented in arable loam. Although it is difficult to ascertain the importance of protozoa, nematodes, rotifers, and myxomycetes in the control of soil fungi, it is certain that the mere survival of an introduced fungus pathogen is more probable in an acid, poorly drained soil than in an arable loam, since the former soil does not permit the development of a large population of animal types which are fungus predators.

Brian (102) considers that the possibility of biological control of soil borne plant diseases merits extensive study. This opinion might well be advanced for the control of soil-inhabiting pathogenic fungi which cause systemic mycoses. If adequate therapeutic measures for the treatment of the several systemic mycoses are not forthcoming in the near future, the increasing importance of mycotic fatalities (Fig. 1) may well necessitate control of this fungus reservoir.

ISOLATION AND IDENTIFICATION METHODS

Many reviews have appeared recently treating the problems of isolation and identification of pathogenic fungi, mostly from the viewpoint of the

diagnostic laboratory (103, 104, 105). The increasing attention that is being directed to the public health aspects of fungus infections will, no doubt, bring about much work on diagnostic procedures.

The effects of four different concentration procedures commonly employed for the isolation of the tubercle bacillus were examined with fungi causing pulmonary mycoses (106). Most pathogenic fungi did not survive any of the isolation procedures. However, *Nocardia asteroides* withstood isolation from sputum when trisodium phosphate was employed as digestant, and *C. albicans* withstood the sulfuric acid digestion method.

The incorporation of antibiotics in culture media for the selection of certain microorganisms has been recommended for many years, and it has become commonplace to include antibacterial agents in media for selective isolation of pathogenic fungi. More recently, with the discovery of antifungal antibiotics, it has been possible to select substances whose antifungal spectrum includes common, saprophytic molds but against which a given pathogenic fungus may, perchance, be more resistant. Thus, incorporation of $C_{15}H_{23}NO_4$ (Actidione) (0.1 mg. per ml.) in an agar medium has been reported (107) to give a selective medium for the isolation of *C. immitis*. This medium may be suitable for the attempted isolation of *C. immitis* from air. Other media recommended for use as primary isolation media include blood agar for dermatophytes (108), brain-heart infusion, blood agar for *H. capsulatum* (109), chocolate agar for *Paracoccidioides brasiliensis* (110), and a glucose-glycine-yeast extract medium containing bismuthyl-hydroxysulfite for the isolation of *Candida* (111). On the latter medium bacteria and most molds do not develop, whereas species of *Candida*, including *C. albicans*, *C. tropicalis*, and *C. krusei*, make black growths which are distinctive.

Selective methods for *in situ* staining of fungi in tissues have been developed. Kligman & Mescon (112) and Pillsbury & Kligman (113) have applied the Hotchkiss-McManus periodic acid-Schiff stain for the localization of fungi in tissues. Techniques for rapid permanent staining and mounting of fungi in skin scrapings and hair have been described (114, 115). Through the use of a synthetic detergent (sodium lauryl sulfate) it is reported possible to clear skin scrapings for direct microscopic observation and yet permit subsequent inoculation of the specimen into media to obtain the fungus in culture (116).

Maintenance of cultures of fungi under sterile mineral oil is standard practise in many laboratories as a result of the demonstration by Raper & Alexander (117) and Buell & Weston (118) of the value of the method. The viability of various pathogenic fungi conserved in culture by this method has been reported by Ajello *et al.* (119), and Herrero (120) has studied the conservation of fungi in glass ampules.

Several slide culture techniques have been introduced (121, 122), and observation of the development of fungi contained in infected hairs and skin scales after transfer to a hanging-drop preparation has been recommended

by Raubitschek & Sagher (123) as an adjunct to routine culture procedures. This technique was employed some years earlier by Davidson & Gregory (124) in their studies on *in situ* cultures of dermatophytes. Vogel (125) has described a modified van Tieghem mount that should prove useful in such studies. Vanbreuseghem (126) has carried further the studies of Davidson and Gregory, confirming their well known observations on the development of macronidia of dermatophytes on hair in isolated cultures. In no instance, out of 200 tests on isolated hairs inoculated with dermatophytes, did he observe the fungus to assume its normal, parasitic appearance. Rather, he has found isolated hair-cultures of *Trichophyton* (*Ctenomyces*) *persicolor*, and 2 other dermatophytes, to form structures invading the hair. These structures he has termed "organes perforateurs"; they resemble in appearance the haustoria of some phytopathogenic fungi. This work is illustrated with excellent photographs.

The influence of nutrition on chlamydospore formation in *C. albicans* has been the subject of several studies. Incorporation of a strain, known to produce chalmydospores, into each series of unknown strains tested on corn meal agar again has been recommended (127). A medium of known composition containing soluble starch, freed from glucose and maltose, has been found in several laboratories to be well suited for chlamydospore formation (128, 129). The presence of glucose in corn meal agar has been stated to be a deterrent to chlamydospore formation (130).

EXPERIMENTAL INFECTIONS WITH FUNGUS PATHOGENS

Animal infections.—Difficulties in the production of experimental infections in animals with agents of the systemic mycoses are typified by the report of Howell (131). Of 80 guinea pigs inoculated intraperitoneally with large doses of yeast phase cells of *H. capsulatum* only 3 developed a generalized infection, and only 17 of 82 animals exhibited a generalized infection when similarly injected with the yeast phase of *Blastomyces dermatitidis*. Cultures of either organism could readily be obtained from the spleens of apparently healthy animals that had been inoculated several months previously, although no gross lesions on the spleen were visible at autopsy.

Uniform and rapidly fatal infections in mice are reported by Heilman (132) to result from intravenous injection of either the yeast phase or mycelial phase of two different cultures of *B. dermatitidis*. The time of death ranged from 7 to 38 days for this route and was directly related to the number of organisms administered, being similar for both culture phases of the fungus. Death resulted in 38 days from the injection of as few as nine cells in the yeast phase. The disease process in mice appeared to be similar to that following the intravenous injection of large doses of virulent human strains of *M. tuberculosis*, consisting of an extensive involvement of the lungs with death resulting from embolic pneumonia. Intraperitoneal injection of *B. dermatitidis* appears to have resulted less regularly in fatal infections; however, in-

jection of large doses by this route has been reported by some workers to yield fairly uniform results (133, 134), although with somewhat greater variation in the time of death than normally follows intravenous injection.

Fatal infections in young mice are reported by Parsons (135) to result from intravenous injection of massive doses of *H. capsulatum*. The generalized disease was characterized by a pronounced enlargement of the spleen, a feature not observed in experimental blastomycosis in mice (132).

Infection of the hamster with the causative agent of South American blastomycosis, *P. brasiliensis*, has been studied (136), and infections of guinea pigs and mice with *Paracoccidioides* (*Glenosporella*) *loboi* (causative agent of Lobo's disease, or cheloid blastomycosis) have been reported (137). The organism, grown at 37°C. on blood agar, showed spherical cells multiplying by single or multiple budding (138). Similar forms were observed in purulent exudates from testicular lesions of experimentally infected animals.

Strauss & Kligman (139) and Campbell & Saslaw (140) found that the resistance of mice to infection by agents of the systemic mycoses was markedly reduced when the infecting fungus was suspended in 5 per cent gastric mucin. Fatal infections were produced with most of the fungi tested. The enhancement of virulence of the infecting agent or the weakening of host resistance prompted by gastric mucin has been confirmed by other workers for *C. albicans* (141) and for the isolation of *H. capsulatum* from soil (93).

Tissue culture.—The growth of *H. capsulatum* in tissue culture has been recorded (142, 143), and cultivation of *C. albicans* in chicken plasma clotted by chick embryo extract has been recommended for the rapid detection of chlamydospore formation (144).

Infections in embryonated eggs.—Inoculation of *C. immitis* into the yolk sac of embryonated eggs has been claimed by some workers to result in conversion of arthrospores directly into the spherules characteristic of the tissue phase of coccidioidomycosis (145, 146); this has been disputed (147). Vogel & Conant (146) report being able to harvest the spherules from egg yolk by a differential centrifugation technique. Inoculation via the chorioallantoic route has been employed (148, 149) but is said to be somewhat less satisfactory than yolk sac inoculation for the recovery of masses of spherules. Although *C. immitis* has been reported to develop mycelial growth on the chorioallantoic membrane (148), others (146) find no evidence that the fungus grows in this form in yolk sac tissue. Mortality of embryos infected with *C. immitis* is usually low, and although hatched chicks may be found to be heavily infected with *C. immitis*, they apparently suffer no ill effects. Newcomer, Wright & Tamblyn (147) could find no evidence that the embryonated egg supported massive growth of *C. immitis* in the spherule form. The fungus was found to exist in the yolk sac and allantoic cavity in the form of loose mats composed of hyphal elements. They comment on the superficial resemblances between yolk cells and the spherule form of *C. immitis*.

Infections in embryonated eggs have been studied as a means of obtaining

a type of *in vivo* evaluation of antifungal substances. Action of four anti-biotics on a nonfatal infection of the chorioallantoic membrane, caused by injection of the yeast phase of *B. dermatitidis*, was examined (150). Aggrava-tion of the lesions by small doses of streptothricin was observed. Criteria of chemotherapeutic effectiveness in egg infections are not numerous and, in general, nonfatal infections have been studied with difficulty. The single cri-terion of prevention of death has proved most reliable; delay of time of death has proved to be less satisfactory for the evaluation of antifungal action.

Multiplication of *P. brasiliensis* in embryonated eggs infected via the chorioallantoic route has been demonstrated by Monteiro, de Almeida & de Almeida Moura (151). The yeast form of the fungus was obtained from granulomatous lesions of the chorio allantoid and visceral allantoid mem-branes. The pathogenicity of *C. albicans* for embryonated eggs has been stud-ied by several workers (152, 153).

Manifestations of Fungus Infection after Antibacterial Therapy

Subsequent to the use of oral penicillin, pharyngeal infections with *Candida* have been reported (154) to be of frequent occurrence, although secondary infections by fungi have rarely been observed to follow the paren-teral injection of penicillin or streptomycin. With increased use of oral ther-apy, employing particularly aureomycin, chloramphenicol or terramycin, infection with *Candida* has been reported with increasing frequency, espe-cially in association with gastroenteritis and vitamin B deficiency symptoms (155, 156, 157). The frequency with which mucocutaneous lesions, including stomatitis, perlèche, and vulvovaginitis have appeared following aureomycin therapy has been attributed by some workers (154) to an avitaminosis re-sulting from suppression of the bacterial flora of the intestine. Unusual fun-gus infections, such as endocarditis due to *Candida* or *Aspergillus*, also have been reported as a consequence of antibiotic therapy (158, 159). Avitamino-sis has been viewed as a major factor predisposing to *Candida* infection, and cases of mild infection of the upper respiratory tract from which *C. albicans* had been isolated are reported to respond favorably to vitamin B complex and liver extract administered orally or by injection (160). Reiches (161) considers a loss of tissue resistance to invasion by *Candida*, subsequent to antibiotic therapy, as likely to be a consequence of tissue changes resulting from a sensitization reaction. An overgrowth of *Candida* on perianal skin was observed in an individual, sensitized to chloramphenicol, following direct application of the antibiotic to the perianal skin.

The methyl ester of paraben (*p*-hydroxybenzoic acid) has been reported to inhibit the growth of filamentous fungi and the propyl ester delays the growth of yeasts, including *C. albicans* and *C. kruesi* (162, 163). These rela-tively nontoxic substances have been found (162) to be of some value in preventing the overgrowth of *C. albicans* after oral therapy with aureomycin,

but do not appear to possess much activity when antibiotic therapy is administered rectally or vaginally.

Incidence of Candida in mouth flora following antibacterial therapy.—C. albicans has been reported to constitute part of the normal mouth flora in 6 to 30 per cent of the individuals examined in different surveys (164, 165). Lipnik, Kligman, and Strauss (164) found a normal incidence of 7.5 per cent for *C. albicans* in mouths of individuals before antibiotic therapy. The frequency jumped to 80 per cent for patients receiving aureomycin or chloromycetin and, of the species isolated, approximately 80 per cent proved to be *C. albicans*. Accompanying the increased frequency of *Candida* was a very marked increase in the numbers of colonies of *Candida* obtained in each isolation. Species of *Cryptococcus* and *Geotrichum* were occasionally isolated. Despite the increased prevalence of *Candida*, the authors observed only one clear cut case of clinical thrush. Therefore, they sound a warning note that the mere isolation of *Candida*, in the absence of correlated clinical findings, is insufficient evidence on which to allege a causal relationship between antibacterial therapy and an increase of mycotic infection. The review by Kligman (166) is especially pertinent in this connection.

*Stimulation of fungus growth by antibacterial substances.—*The apparent decrease in resistance to *Candida* resulting from the administration of antibiotics has been subjected to experimental study by Seligmann (167). A strain of *C. albicans* which was nonpathogenic for mice was found to cause a fatal infection when aureomycin and the yeast were injected intraperitoneally. The potentiating effect of aureomycin was observed when it was administered from 24 hr. before to 4 hr. after infection with *C. albicans;* if given 8 to 24 hr. after infection, no effect was observed. The adjuvant action of aureomycin was lost in parallel with loss of its antibacterial action as a result of boiling.

These results are challenged by Kligman (164, 166) who has been unable to observe any potentiating action of antibacterial agents on experimental infections with *C. albicans, B. dermatitidis, H. capsulatum, C. immitis,* or *Sporotrichum schenckii* in mice. Campbell & Saslaw (140) previously had failed to find potentiation of *H. capsulatum* infection in mice treated with streptomycin. But, to offset these reports, Brown, Hazen & Mason (168) state that injections of aureomycin and *C. albicans* into mice resulted in the death of all animals within 24 hr. whereas only 70 per cent of the mice receiving the organism alone died (and these only within 2 to 12 days) and 30 per cent were still alive at 16 days. Fungicidin, given together with the aureomycin-yeast combination permitted all mice to survive. This report confirms and extends the work of Seligmann.

Reports (169, 170) of the stimulation of growth of *C. albicans* by preparations of aureomycin employed for oral administration (not with crystalline aureomycin hydrochloride) have been shown conclusively to be attributable to phosphate ions present in the inert diluent of the oral preparation (164).

The nutritional requirements of species of *Candida* were investigated some years ago by Burkholder & Moyer (171, 172) who showed that most strains of *C. albicans* on a synthetic glucose-salts-ammonium medium require only the addition of biotin for maximal growth. It was shown that *C. krusei* similarly required biotin and was stimulated by thiamine, but liver extract did not stimulate in the presence of optimal biotin and thiamine. Paine (173) purports to demonstrate the failure of *C. krusei* to make any detectable growth on a mineral salts-glucose agar medium (agar not extracted) or on this medium after the addition of 0.5 mg. per ml. of yeast extract (a fairly good source of biotin and thiamine), whereas growth appeared on the addition of a vitamin mixture. These results must be considered anomalous in view of former studies on the nutrition of *C. krusei*.

Enhancement of growth of laboratory strains of several filamentous pathogenic fungi in a mineral salts-glucose liquid medium on adding increasing concentrations of streptomycin has been reported (174). Only qualitative observations of fungus growth were supplied, and the evidence for growth enhancement is based on macroscopic comparisons which are notoriously difficult and frequently misleading. The medium employed contained no added growth factors and was adjusted to a final pH, scarcely optimal, of 4.0. The streptomycin effect was maximal at 2.5 mg. per l. Strains of some of the organisms examined in this study, *S. schenckii*, *P. verrucosa*, *T. mentagrophytes*, and *H. capsulatum*, are known to possess a requirement for thiamine (175). Moreover, the requirement of normal (nonpleomorphic) *T. mentagrophytes* for an amino nitrogen source is well documented. These qualifications raise some question concerning the nature of the strains covered by this report and the reality of the phenomenon reported.

CHEMOTHERAPY OF FUNGUS INFECTIONS

Chemotherapy of fungus infections is being studied in a great many clinics and laboratories; while knowledge on the subject is being accumulated rapidly, much of it is difficult to assess. In the following section there will be presented simply a selection of three developments among the scores of lines being pursued in chemotherapeutic investigations.

Fatty acids.—The employment of fatty acids for therapy of mycotic infections was originally proposed by Peck & co-workers (176). Interest in the fungicidal properties of the fatty acids (including propionates and esters thereof, caprylate, pelargonate, and undecylenate) for the treatment of dermatomycosis has been maintained (177, 178). Growth of *C. immitis* has been reported (179) to be completely inhibited by sodium caprylate (less than 150 μg. per ml.). Sodium caprylate has also been reported to be effective in the treatment of clinical thrush in children (180, 181), and propionate appears to be effective in treatment of vaginal infections from which *Candida* had been isolated (182).

An interesting study has been reported (183, 184) relating the appearance

of *M. canis* infection in cats to nutrition. Experimental animals fed a purified diet deficient in certain aspects rapidly developed lesions which disappeared when natural foods were restored to the diet. Evidence was advanced that *M. canis* existed saprophytically in the skin but developed pathogenicity only when the animal was placed on a restricted diet. The relationship between restricted dietary regime and pathogenicity is obscure but may operate through a diminished formation of skin fatty acids (and thus be a question more of decreased host resistance than of increased virulence of the fungus). The authors see a relationship between their work and that of Rothman (185) on the pelargonic acid content of the skin of children versus that found in the skin after puberty.

Diamidines.—The observation of Elson (186) that certain pathogenic fungi were inhibited by low concentrations of propamidine [p,p′-(trimethylenedioxy) dibenzamidine] has centered interest in the treatment of fungus infections with the diamidines. Propamidine has been used as an adjunct to ethyl vanillate (ethyl 4-hydroxy-3-methoxy benzoate) in the treatment of histoplasmosis (187). The efficacy of stilbamidine (4,4′ stilbenedicarboxyamidine) in the treatment of blastomycosis (188, 189) and in treatment of actinomycosis (190) has been reported by Schoenbach, Miller & Long. However, stilbamidine has been found to have no effect on experimental infections of *C. neoformans* in mice (191). The chemistry and pharmacology of diamidines has been reviewed (192) by Schoenbach & Greenspan.

Antibiotics.—As Waksman has pointed out, some antibiotic substances (including gliotoxin, clavacin, actinomycin, streptothricin, and tyrothricin), are active on both bacteria and on fungi, some are active only against bacteria and actinomycetes, and others [including actidione, antimycin, fradicin, fungicidin, and candicidin] are active primarily against fungi (193, 194, 195). Antifungal antibiotics have been compared on the basis of origin, spectrum, and chemical nature [Waksman *et al.* (195, 199)]. The antifungal agents which have been described recently include actidione (196), fradicin (197), fungicidin (198, 199), rimocidin (200), and candicidin (201).

Fradicin and thiolutin, both in a concentration of 13 μg. per ml., were found to inhibit completely the growth of *C. immitis* on a Sabouraud-type agar (179). Actidione inhibits the growth of *C. immitis* but has proved toxic, and the same can be said for protoanemonin which is markedly inhibitory *in vitro* against *C. immitis*. *Geotrichum* septicemia in a diabetic man has been reported following a pharyngitis caused by *C. albicans* (202). *Geotrichum* species was isolated from blood and from sputum. Neomycin proved effective in treatment of the septicemia, although toxic reactions were manifested toward the antibiotic. This substance has also proved effective against *Geotrichum* infection of the urinary tract that had not responded to streptomycin or aureomycin (203).

PHYSIOLOGY OF PATHOGENIC FUNGI

Growth factor requirements.—Since the subject was reviewed in 1947 (204), an extensive literature has accumulated. Area Leao & Cury (175) examined the requirements of 60 strains representing a large number of species. Of these, 35 exhibited no growth factor requirement on a synthetic, purified agar medium with recrystallized asparagine as source of nitrogen. A deficiency in thiamine synthesis was found in 17 strains, including two strains of *P. brasiliensis,* and one of *H. capsulatum.* An interesting finding was the biotin requirement of two strains of *A. boydii,* later studied in detail by Villela & Cury (205). Salvin (206) has mentioned the biotin requirement of one strain of *H. capsulatum,* but otherwise a requirement for biotin appears not to occur among filamentous fungi and is a characteristic deficiency of most yeasts. Niacin has been shown to be required by a strain of *M. audouini* (175).

Mackinnon & Artagaveytia-Allende (207, 208) found that among 5 strains of *T. favifome* (*discoides*) studied in a synthetic glucose-asparagine medium, one required thiamine, another needed thiamine, pyridoxine, and inositol for growth (in agreement with an earlier report), and three strains required only i-inositol. One strain of *T. faviforme* (*ochraceum*) needed inositol and another both inositol and thiamine. The authors did not feel that the strain differences warranted specific differentiation and advanced the opinion that the biochemical differences among morphologically similar strains might represent recognition of naturally occurring mutants (208). This is an interesting possibility inasmuch as numerous isolations of "wild type" *Neurospora* have not turned up any of the multiplicity of biochemical mutants that have been induced by irradiation. The large spored faviform *Trichophytons* of animal origin may be perpetuated in a natural environment which is not so stringent in the elimination of mutants lacking especial survival value. In agreement with other investigators (175, 208), Georg (209, 210) considers as synonyms the Sabouraud types *T. album, T. discoides,* and *T. ochraceum* and groups them into a single species *Trichophyton faviforme.* Demonstration that all three types can be obtained from a single spore isolation supports the data obtained from nutritional studies. The latter studies had indicated that the group was distinct from *T. schoenleinii* which exhibits no growth factor requirements in a synthetic medium (175, 209).

Lipophilic yeast myco flora of the skin.—The nutritional requirements of *Pityrosporum ovale,* commonly isolated from the scalp, were surveyed by Benham (211) in 1947. The lipid requirement of this yeast is met by a long chain unsaturated fatty acid such as oleic acid (211, 212). Gordon (213, 214) has recently isolated from glabrous skin several strains of a yeast, akin to *P. ovale,* which he has designated *P. orbiculare.* This organism requires a fatty substance and grows well on a Sabouraud type agar to which various natural

oils or a long chain saturated fatty acid had been added. Unsaturated fatty acids such as oleic or linoleic did not permit growth. The new species is further distinguished from *P. ovale* by the narrow isthmus connecting bud with parent cell in *P. orbiculare*, in contrast to the characteristic budding on a broad base exhibited by *P. ovale*. This morphological difference has caused Gordon to redefine the genus *Pityrosporum* to permit the inclusion of morphologically different species related by their lipophilic character. The merit of this decision may be open to question since the morphological criterion of budding on a broad base has been taken to be a valid basis for generic classification of yeasts (although in most genera separated on this basis the budding is bipolar as well). The requirement of *P. ovale* for an unsaturated fatty acid probably rests on an entirely different biochemical basis from the requirement of *P. orbiculare* for a saturated fatty acid. It is not improbable that the isolates termed *Pityrosporum orbiculare* may prove to be cultural forms of the apparently dimorphic *Malassezia furfur*. In this connection Gordon has himself stated (214),

> The most frequently cultured fungus was a thick-walled, spherical, budding organism, 2.1–4.8μ in diameter, very closely resembling the spherical elements of *M. furfur* that appear in lesions. It was recovered only from young, actively spreading lesions, in 13 of 18 cases, and produced no growth on Sabouraud dextrose agar (Difco) without oil. Since it was so consistently associated with lesions of tinea versicolor and so highly suggestive morphologically, of the round forms of *M. furfur*, it was subjected to intensive cultural studies as well as experimental inoculations in human subjects.

The failure of the experimental inoculations to produce tinea versicolor can hardly be taken as definitive evidence against the fungus' being *M. furfur*, especially in view of the many difficulties surrounding experimental infections with fungi. On the basis of this criterion could one establish identity between *P. ovale* isolated in culture with the organism seen in epidermal scales?

Pigments.—Preliminary data on the pigment of *M. gypseum* have appeared (215). It is soluble in aqueous solution, yellow in acid solution, and yellow orange to red brown in alkaline solution, and is insoluble in organic solvents. An orange-red variant of *T. mentagrophytes* has been reported (216). The variant was isolated on several occasions from ringworm of the glabrous skin and nails. Young cultures developed a yellow color that deepened to orange-red as the culture aged and became alkaline. In solution, the water soluble pigment changed color from yellow to orange-red on adding alkali. The statement is made, although no evidence is advanced in susbtantiation, that the orange-red pigment of the variant is "chemically identical to the cream to rose pigment of the normal strain of *T. mentagrophytes;* the striking change in color between the normal and nodular varieties of *T. mentagrophytes* being of a quantitative nature." Since the chemical nature of the pigment of the parent strain has not been elucidated, it is difficult to understand on what basis the authors claim chemical identity of the pigments. Other

studies on pigments include that of Bocobo & Benham (217) on *T. rubrum*, and on the separation of a diffusible pigment from a pigment retained in the mycelium of *A. boydii* [Villela & Cury (218)].

Metabolism of Sporotrichum schenckii.—The utilization of carbon sources by several strains of *Sporotrichum* has been reported [Lurie (219)]. From a study of the nitrogen metabolism of these strains Lurie (220) reports that some appear to be able to fix atmospheric nitrogen. It will be interesting to see whether this finding can be confirmed in other laboratories. The work reported by Lurie appears to have been well controlled, but is based on micro-Kjeldahl determinations on fungus weights of only 3.0 to 9.5 mg., and the amounts of nitrogen fixed are estimated as 0.018 to 0.087 mg. per flask after four weeks incubation in a nitrogen-free medium. The chance for traces of ammonia in the atmosphere to contribute this amount of nitrogen is ever present, unless specially guarded against, and no special mention on this point is included in the report. *S. schenckii* would be an interesting nitrogen-fixing organism, for it utilizes both nitrate and amino acid nitrogen, and a mixture of amino acids was preferred to any single one.

MORPHOGENESIS OF PATHOGENIC FUNGI

Multiple budding of dimorphic fungi.—Salvin (221) obtained the budding, or tissue phase, of seven strains of *S. schenckii* on cultivation in a semisolid glucose-peptone medium of pH 8.2 at 37° in an atmosphere of 60 to 80 per cent CO_2. Two types of multiple budding were observed: a bipolar type, and another in which buds were distributed over the cell surface. The occurrence of multiple-bipolar budding in *C. albicans* has been demonstrated by Nickerson & Mankowski (128, 129) and shown to result from the failure of buds (formed singly) to abcise promptly after their formation. The distinction between multiple-bipolar budding and a multiple-apolar or "over-all" type of budding appears to depend upon the extent of departure of the mother cell from a spherical or subspherical shape (222). Barton (223) has observed the succession of bud forming sites in *Saccharomyces cerevisiae* through the first 15 buds (each bud abcising normally). Each bud formed at a new site on the mother cell, and the succession of bud-forming loci followed a definite pattern. "Bud scars" were demonstrated on the surface of the mother cell and serve to mark the loci of budding. Cells with up to 23 "bud scars" were reported. The distribution of these markers revealed that the loci of budding are not exclusively polar. In *C. neoformans* the "bud scars" are plainly exhibited and have a collar-like appearance as described by Negroni & Negroni (224). In *P. brasiliensis* analogous structures have been described by Mackinnon & Gurri (225). It is obvious that retention of buds on the mother cell (provided it did not elongate) would result in the appearance of multiple-apolar or an over-all type of budding. In yeasts which are prone to form elongated cells, the appearances of multiple-bipolar budding may result from the retention of buds formed at the poles of a cell and interference with

lateral sites of bud formation (which are rarely the initial loci for budding) as a result of elongation of the cell (129, 222).

The importance of a high CO_2 tension for the development of the yeast phase of *H. farciminosus* was shown by Bullen (226) while Drouhet & Mariat (227) confirm the requirement of CO_2 for the M→Y transformation in *S. schenckii*. However, they report 5 per cent CO_2 at 37° to be satisfactory in a synthetic glucose-salts medium containing ammonium sulfate, thiamine, and biotin. Agitated liquid cultures in the synthetic medium with certain amino acids (arginine, for example) developed yeast forms in abundance in a normal atmosphere of air.

Studies on the uncoupling of cellular division from the growth process have been reported for *B. dermatitidis* and *P. brasiliensis* (228, 229). Enzymatic mechanisms for reduction of disulfide links in cystine and in oxidized glutathione have been described in *C. albicans* (230). The importance of maintenance of sulfhydryl groupings for the operation of the cellular division mechanism and the M→Y transformation in *C. albicans* and in certain dimorphic fungi has been reported (231, 232, 233).

Capsulation of Cryptococcus neoformans.—In giant colonies on Sabouraud-maltose agar Drouhet & Couteau (234) observed smooth, rough, and mucous sectors. The smooth form was most stable and comprised cells of 4 to 6 μ in diameter with a thin capsule not more than 1 μ thick. The rough type had no capsule and showed a tendency toward filamentation. Mucous sectors were composed of cells with enormous capsules up to 10μ thick. Maltose favored capsule formation whereas glucose was unfavorable for this development. The capsular material is composed of xylose, mannose, and a uronic acid (235).

Morphogenesis of dermatophytes.—Vanbreuseghem (236) and Vanbreuseghem & Van Brussel (237) have found that inclusion of soil in an impoverished agar medium favored the development of accessory morphological structures in abundance. In two instances reversion of pleomorphic strains to sporulating type has been observed following cultivation on a soil-agar medium (238). These reversions were obtained with strains that had recently mutated to the pleomorphic form. No reversion was obtained with pleomorphic forms that had been maintained in culture for many years. Since relatively large inocula were used in this study, it is possible that included in the supposedly pleomorphic cultures were some elements of the parent type whose growth may have been favored by the soil medium. No assurance has been given by these authors that the pleomorphic strains, in which reversion was obtained, had been transferred frequently in the rapidly growing pleomorphic form. Reversion of pleomorphic type to parent sporulating form is a highly unusual occurrence. This work deserves to be studied in other laboratories.

On the impoverished medium formed by solidifying soil with agar, Vanbreuseghem and Van Brussel (237) report the appearance of spirals among the accessory morphological structures developed by *Epidermophy-*

ton floccosum. These structures are rarely, if ever, seen on the richer media commonly employed for the culture of dermatophytes. The structures had been figured by Langeron & Milochevitch (239) for a culture on barley grain, and recently have been described by McCormack & Benham (240) from cultures on corn meal agar. It will be recalled that in 1937 Davidson & Gregory (241), in their study on comparative aspects of spiral hyphae formation in dermatophytes, recommended a 1 per cent whole wheat flour agar. Soil agar, barley grain, and corn meal agar media may be expected to be poor in sugar. It will be of interest to learn if the addition of glucose or maltose to these media suppresses the appearance of spirals and other accessory morphological structure, as these rapidly metabolized carbohydrates have been found to suppress the development of filaments and chlamydospores in *C. albicans.* (128, 129). It is very difficult to discern the underlying principle, if one indeed exists, relating to the morphogenesis of *E. floccosum* on soil agar or on corn meal agar, and to the reports by Georg (242, 243, 244) of the necessity for using rich media with added thiamine for the development of macronidia in *T. faviforme* and in *T. violaceum.* As Schopfer (245) points out, vitamins do not act as specific morphogenetic factors in fungi but affect morphogenesis generally through participation in metabolic reactions involved in growth. It is clear that in the dermatophytes the biologist has a wealth of types which may furnish excellent material for attempted analysis of the physiological bases of morphogenesis.

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AMEBIASIS[1]

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Introduction

The present status of research on amebiasis tempts one to suggest that the time is not ripe for a review of the field, and, indeed, the dearth of such reviews intimates that this feeling is widespread. Present knowledge of amebiasis is factually very disappointing. New information of fundamental significance is scanty, and the profusion of detailed data can scarcely be summarized. A review simply of facts would certainly be inappropriate at this time.

Certain characteristics of the field, however, are worthy of scrutiny, for they portray the struggle of a scientific discipline to rise out of the confusion of technology into a state where methods are adequate for the revelation of fundamental knowledge. All sciences go through this primary technological phase. The study of amebiasis has not fully emerged from it. Certainly, there have been important discoveries on the biology of the parasitic amebae, and these we shall attempt to outline. Just as certainly, the outstanding feature of the field is its methodological poverty.

The present review, then, will be primarily an effort to delineate those problems which most seriously are engaging the attention of workers on the parasitic amebae of man. It will be a summary rather of questions than of answers, an attempt to show what students of amebiasis are trying to do and why and to indicate what they need to know in order to solve the most pressing problems in the area. With this as the principal emphasis we shall, however, not neglect the stubborn, and partly successful, attempts to utilize available methods for whatever information they can provide.

The latest comprehensive review of amebiasis is Craig's primarily clinical monograph of 1944 (38)[2]. Because developments since 1944 in the morphological and clinical features of amebiasis are considered less important for our purposes than other aspects of the field, these areas are excluded from the present review. Further, in keeping with the primary intention of depicting the present state of the field, treatment is limited largely to publications of the last five years, and, in general, earlier work is referred to only if it contributed to an understanding of current developments. Otherwise, the principal emphasis is given to two subjects which seem to exhibit the most important unsolved problems, artificial cultivation and virulence.

Amebiasis is the condition of infection with *Entamoeba histolytica* [*Enda-*

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[1] The survey of literature pertaining to this review was concluded in January, 1953.

[2] Reference should be made to the book by Anderson *et al.* (2a), which was published after completion of this review.

moeba of some American workers, see (86)]. This is the only commonly pathogenic human parasite of the family Endamoebidae (class Sarcodina, phylum Protozoa). The active stage is an amoeboid organism, 6 to 60, usually 15 to 35, microns in diameter. This "trophozoite" stage normally inhabits the lower intestine of man and some other mammals, where it may produce or contribute to the production of erosive lesions. Less frequently it appears in necrotic abscesses in other organs, particularly the liver. Under certain circumstances the amebae form cysts, characterized by a resistant wall and an ultimate content of four (rarely more) nuclei. These cysts are the infective stage for new hosts, in the intestine of which they hatch to release a number of minute trophozoites.

Entamoeba coli, Endolimax nana, Iodamoeba bütschlii and *Dientamoeba fragilis* normally exist as commensals in the human intestine, although in rare instances disease has been attributed to *I. bütschlii* (42, 157). *Entamoeba gingivalis*, a common commensal of the mouth, has been blamed for pulmonary suppuration in exceptional circumstances (145).

Three species of doubtful status should be mentioned. *E. dispar* and *E. hartmanni* are considered in the section on *Virulence*. *E. polecki*, a species resembling *E. histolytica* but producing uninucleate cysts with characteristic inclusion masses, is found in pigs and goats. Kessel & Johnstone (82) reported its presence in man. Lubinsky (90), finding cysts of the *polecki* type associated in man with *histolytica*-like trophozoites, supposed that they were aberrant cysts of *E. histolytica*. He admitted, however, that *E. polecki* in man might be a distinct species but recognizable only in the cyst stage. This possibility acquired some support from the observation of Kessel & Kaplan (83) that in monkeys treatment with certain drugs was much more effective against *E. histolytica* than against *E. polecki*.

While this review is focussed on the amebae of man, and particularly *E. histolytica*, several amebae of other animals will appear in the discussion. *E. terrapinae* of turtles and *E. invadens* of snakes, both very like *E. histolytica* in a number of respects, have been exploited by some investigators primarily for the light they may cast on the biology of *E. histolytica*. *E. muris* of rats, mice, hamsters, etc. (110) is of interest both for itself and because it complicates the study of *E. histolytica* in experimental animals. It shares many characteristics with *E. coli*, but its physiological distinctness is evinced by the fact that Neal failed to infect rats with *E. coli* from man (110).

CULTIVATION

The most serious impediment to productive biological study of *E. histolytica* is the want of a practical pure culture method. While some steps have been made, and vigorous study currently is being devoted to this key problem, progress has been frustratingly slow. The principal substrate for cultures of *E. histolytica* is still, as in the pioneer work of Boeck & Drbohlav (17), other microorganisms.

Boeck and Drbohlav used a diphasic medium consisting of coagulated

egg slants overlaid with serum and Locke's solution. In the presence of reproducing bacteria the amebae grew abundantly. The principal improvement in this medium was made by the addition of rice starch, which the amebae ingest readily [Dobell & Laidlaw (47)]. Other modifications have omitted the serum, substituted Ringer's for Locke's solution, or used serum instead of egg slants (47). Cleveland & Collier (33) used liver infusion agar slants overlaid with serum-saline. Nelson (113) introduced slants of agar containing alcoholic extracts of egg or mammalian tissues and covered with saline solution. Balamuth (8) developed a monophasic medium consisting of buffered aqueous egg yolk infusion, to which liver extract was sometimes added. Many other media have been described, including some with most of the ingredients known; these will be discussed in connection with the nutritional requirements of amebae. With the partial exceptions noted below, they serve principally to support the microorganisms which, in turn, act as the main substrate for amebic growth.

Monobacterial cultures, consisting of amebae with a single bacterial associate, have received much study. Five general methods have been employed for obtaining amebae free from microbial associates for inoculation into cultures with the selected bacterium: selection of cysts free from bacteria with a micromanipulator [Rees *et al.* (125)]; chemical sterilization of cysts (see below); antibiotic treatment of cultures and addition of an antibiotic-resistant bacterium; inoculation with trophozoites from bacteriologically sterile liver abscesses [Cleveland & Sanders (34)]; and inoculation of trophozoites from cultures with *T. Cruzi*, which dies out under the new cultural conditions (see pp. 276–277).

Cysts from some strains of *E. histolytica* have been freed of bacteria by treatment with mercuric chloride, potassium permanganate, hydrochloric acid, acriflavine, or by various combinations of these agents (12, 13, 121). While the cysts are more resistant to these substances than are many bacteria, some workers have found the cysts less resistant than the sporulating bacteria present in certain cultures. Dobell (46) described elimination of the spore-forming anaerobes from such mixtures by continuous cultivation with gentian violet in media preconditioned with the desired new associate.

The results of a number of studies of monobacterial cultures are summarized below (12, 13, 31, 34, 45, 46, 70, 74, 121, 125, 139). When possible, specific names from Bergey's Manual (22) are given. In those cases where the applicable current name could not be ascertained, quotation marks are used.

Amebic growth has been reported with each of the following: *Pseudomonas coronofaciens, Vibrio comma, Neisseria catarrhalis, Streptococcus fecalis, S. liquefaciens, S. equinus, S. "hemolyticus," S. "viridans," Escherichia coli communior, Aerobacter cloacae, Klebsiella pneumoniae, Serratia marcescens, Salmonella typhosa, S. schottmuelleri, "Bacteroides sp.," Bacterium stewartii, B. zenkeri, Bacillus brevis, B. subtilis* var. *niger, "B. mesentericus," Clostridium perfringens, "Actinomyces muris,"* and "organism t" [originally designated

"*Leptotrichia buccalis*," a micro-aerophilic, allegedly gram-negative, spore-forming rod (126)]. These species vary greatly in their growth-promoting capacities. *C. perfringens* and "organism t" are said to support unusually luxuriant growth of *E. histolytica*.

The following have failed to support amebic growth: *Pseudomonas aeruginosa, P. striafaciens, Xanthomonas translucens, Micrococcus pyogenes* var. *albus, Corynebacterium diphtheriae, Alcaligenes fecalis, Escherichia coli* var. *acidilacti, Salmonella* spp., *Shigella dysenteriae, S. paradysenteriae, Brucella suis, B. abortus, Bacillus megatherium,* and *B. cereus.* Three yeasts also have failed to support growth: *Saccharomyces cerevisiae, Torula histolytica,* and *Monilia albicans.*

Conflicting data have been recorded for the following bacteria, both successes and failures having been reported: *Micrococcus pyogenes* var. *aureus* [pos. (125); neg. (46)], *Aerobacter aerogenes* [pos. (12); neg. (31)], *Proteus vulgaris* [pos. (13, 34, 46); neg. (31)], *Salmonella paratyphi* [pos. (74); neg. (31)], *Bacillus subtilis* [pos. (31); neg. (34)], and *Escherichia coli* [pos. (12, 13, 46, 74); neg. (31); variable (125)]. The findings of Rees *et al.* (125) with respect to *E. coli* offer one explanation for the discrepancies cited. They observed amebic growth with one strain of *E. coli,* while with several other strains multiplication did not occur. Differences in strains of amebae, media, and other factors may also contribute to the disagreements.

Information is disappointingly meager on the properties which enable bacteria to support growth of amebae. Chang (29) and others have postulated several ways in which the bacteria may act, principally by (a) alteration of physical conditions, such as oxidation-reduction potential or pH, (b) production of essential nutrients, and (c) provision of necessary enzyme systems. In monobacterial culture studies Balamuth & Wieboldt (13) observed a general correlation between vigor of amebic growth and the maintenance of a low oxidation-reduction potential. These investigators and Chinn *et al.* (31) noted also that the most favorable bacteria proliferated only moderately in the amebic media used. The injurious effects of *S. fecalis* in glucose-containing media (144), of *A. fecalis* (46), and of *C. sporogenes* (31) indicate that some of the unfavorable bacteria manufacture substances toxic for the amebae. Evidence that growth-supporting bacteria contribute to the nutrition of *E. histolytica* is discussed below.

Extensive study with polybacterial cultures of known composition particularly by Dobell (46), Chinn *et al.* (31), and Balamuth and collaborators (9, 12, 13) cannot be summarized effectively. Balamuth & Wieboldt (13) have given particular attention to the dynamics of populations in both mono- and poly-bacterial cultures. The interrelationships between bacterial and amebic growth curves are obvious but complex, and it is clear that only a better understanding of the physiological dependences of amebae upon their microbial associates will permit elucidation of the ecologies involved.

Trypanosoma cruzi is the only microorganism aside from bacteria which has been utilized as a substrate for *E. histolytica.* Phillips (115) observed

growth of amebae in a sealed thioglycollate medium with added living trypanosomes. He later obtained inferior growth with *Strigomonas fasciculata*, slight success with *T. conorhini*, and none with *T. pipistrelli* or three species of *Leishmania* (116). Rees (124) similarly failed to cultivate amebae with *Trichomonas foetus*. Heat treatment sufficient to inhibit respiration of the trypanosomes (52°C.) destroyed their growth-promoting properties, while heating which merely inhibited reproduction (48°C.) did not (108, 118). The amebae seem to acquire a marked physiological adaptation to growth with *T. cruzi*, for Nakamura (105) failed to reassociate them with normally favorable bacteria, and Luttermoser & Phillips accomplished the replacement only after numerous passages (92).

Recent work gives promise of imminent accomplishment of pure culture of *E. histolytica*. Some success has attended efforts to eliminate other active microorganisms from the cultures, and a beginning has been made in determining what it is that the bacteria must furnish for amebic growth. Jacobs (73) described growth in the presence of heat-killed or penicillin-inhibited *Escherichia coli*. Multiplication of the amebae was scanty, but the cultures were maintained for three and one half to six months. Shaffer & Frye (139) developed a method for continuous cultivation with streptobacilli (probably *Bacteroides* sp.) inhibited, although not killed, by penicillin. With this procedure several strains have been maintained for more than three years (137). Sadun *et al.* (133) achieved like results with antibiotic-suppressed bacteria in early embryonic fluids. To date, attempts to isolate and characterize the growth-promoting substances provided by the bacteria have met with failure [Karlsson (80); Karlsson *et al.* (81); Shaffer (137)]. They could not be separated from the bacterial bodies by filtration or by extraction with a variety of solvents. Starvation of the bacteria depleted their growth-enhancing properties (80), and this, together with data on the effect of heat, drying, etc., suggested that a labile, nonprotein metabolite is involved.

Rees and his co-workers (128) have taken a different approach to the problem of pure culture of *E. histolytica*. Having confirmed the observations of Snyder & Meleney (141) and others that excystation of *E. histolytica* could take place in the absence of bacteria if the oxygen tension was lowered, they have examined the influence of various media on the proportion of cysts hatching and the longevity of the excysted amebae. Microisolated, bacteria-free cysts hatched well in a cysteine salt mixture, still better when glucose, vitamins, organic bases, and amino acids were added. In this latter medium survival of the metacystic amebae was prolonged significantly beyond that in inorganic media. Glucose salt mixtures supported moderate survival. Although multiplication of the amebae was not observed in these studies, data obtained in this way can contribute to an understanding of both the nutritional requirements of *E. histolytica* and the necessary conditions for pure culture.

Actually, pure culture of a parasitic ameba has been described. Lamy

(87), with *E. invadens*, reported sparse growth of amebae in the presence of mashed chick embryo or snake tissues. Details are wanting on the procedures and on the degree of permanence of the cultures, but it appears that some success may have been attained in cultivating this reptile parasite free of other microorganisms. Shaffer & Sienkiewicz (140) have recorded propagation of *E. histolytica* for 10 passages with minced or ground chick embryo tissues. Living tissue cells were evident in the cultures, but it was not clear whether proliferation of these cells took place.

PHYSIOLOGY OF TROPHOZOITES

In addition to the studies summarized above on the nature of the microbial contribution to the support of amebic growth, investigators have concerned themselves extensively with the general nutritional needs of *E. histolytica*. The data have limited value, for they show the requirements of an ameba-microbe association rather than of the amebae themselves. While the ecological significance of such data may be considerable, much caution must be exercised in drawing physiological inferences from them. Thus, the importance of a nutrient required for amebic growth may result from (*a*) its direct nutritional value to the amebae, (*b*) its significance to the microbial associates in their synthesis of essentials for the amebae, (*c*) its protective action against injurious substances in the medium, or even (*d*), as in the case of certain amino acids, its participation in the maintenance of such physical conditions as a favorable oxidation-reduction potential. Hansen & Anderson (59) observed abundant growth of *E. histolytica* with "organism t" in a buffered liquid medium containing trace minerals, 12 amino acids, 10 B vitamins, nucleic acid, cholesterol, and rice powder. In a similar medium Hallman *et al.* (58) found that various proteins and maltose or (with one strain) dextrose could be substituted for the rice starch for propagation of polybacterial cultures, but not of the amebae with "organism t" alone. The protein content of the rice powder was apparently essential to growth, and the whole rice powder was necessary for monobacterial cultures. In general, it may reasonably be supposed that those nutritive needs which acquire prominence when the associated flora are simplified represent direct food requirements of the amebae. Adding to the above data those of Snyder & Meleney (142) and of Rees *et al.* (127), we may conclude that *E. histolytica* requires protein, carbohydrate as mono- or poly-saccharide, several B vitamins, and cholesterol. We must surmise that other requirements are met by the associated bacteria, but it should be recognized that a principal function of these bacteria may consist in packaging food substances in a form permitting their ingestion by the amebae.

A number of specific enzymatic activities have been attributed to *E. histolytica*. Acid phosphatase has been identified cytochemically within the amebae [Carrera & Changus (25)]. Dissolution of starch granules inside the cell is well known and appears to represent actual digestion of starch (67). It seems safe to credit to the amebae themselves biochemical activities such

as these, which have been observed in the cells. Somewhat more caution is demanded, however, in interpretation of data obtained with suspensions of washed amebae, although it may be presumed that most environmental effects are eliminated by the washing. With such methods, hyaluronidase activity has been shown in amebae from host tissues but not in those from cultures [Bradin (18)]. Production of H_2S from cysteine has also been demonstrated [Bradin & Kun (20)]. Finally, tentative conclusions may be drawn from enzymatic activities detectable in cultures of amebae growing with bacteria which do not show the enzymes when grown alone. Thus, amylase has been recognized in cultures of *E. terrapinae* [McCarten & Griffin (94)]. Although the bacterial associates did not produce it, they modified its action, and it must be emphasized that two organisms may exhibit biochemical activities which neither shows by itself. The same reservation is necessary concerning the observation that *E. histolytica* with "organism t" acted on the protein envelope of some rice powders, while "organism t" alone had no such effect [Reardon & Bartgis (122)].

E. histolytica is a strict anaerobe, showing optimum growth at oxidation-reduction potentials below −300 mv. [Jacobs (72); Chang (29)]. The highest potentials permitting growth are not clear. Chang (29) observed no growth at potentials above −200 mv. and rapid death at −50 mv. Jacobs (72) and Balamuth & Weiboldt (13) have recorded some, but inferior, proliferation at potentials up to about −100 mv. Optimum pH ranges of 5.95 to 6.9 and 6.6 to 7.3 have been reported [Chang (30); Shaffer (137)]. Little or no growth was seen at less than pH 5.6 or above 7.0 (137) or 8.0 (30).

ENCYSTMENT

Encystment of *E. histolytica* occurs in three general circumstances. Normally it takes place in the colon contents under conditions in which solid stools are formed. With many cultural methods small numbers of cysts may develop fairly regularly, particularly in strains of recent isolation (44, 47). Finally, certain culture techniques induce a considerable proportion of the amebae to encyst. These techniques commonly involve preparatory cultivation in deficient media, usually without starch, followed by heavy inoculation into exceptionally rich media with an abundance of starch (9, 29, 47). The amebae multiply very rapidly, attaining unusually dense population levels, then, within a few hours, encyst profusely.

In *E. histolytica*, as in other protozoa, crowding and the onset of inimical environmental conditions appear conducive to encystment, but little is known of the specific mechanisms. Chang (29) has shown that the inception of encystment coincides with a rather abrupt rise in the oxidation-reduction potential of a culture following luxuriant growth of the amebae. He obtained sparse production of cysts in cultures aerated to raise the potential artificially.

The influence of the bacterial associates on encystment is noteworthy. Chinn *et al.* (31) found cysts only sporadically in monobacterial cultures.

None have been found by other investigators except for Dobell (46), who observed a few cysts in cultures with a sporulating anaerobe. These cysts appeared abnormal, however, for they could not be induced to hatch. Encystment occurred in cultures with *Escherichia coli* upon the addition of filtrates from cultures of the same anaerobe. Particular bacteria, notably *C. perfringens* (31), and other anaerobic spore-formers (46) have produced profusely encysting combinations when they were added to monobacterial cultures.

There is little information on the factors precipitating encystment *in vivo*. Ordinarily it occurs only in the lumen of the colon, but Radke (120) has reported quadrinucleate cysts in material aspirated from meticulously washed ulcers of the bowel.

Host Range

E. histolytica is a natural parasite of man and certain monkeys, but spontaneous infections occur sporadically also in dogs, rats, and other mammals. Older experimental work mainly utilized dogs, cats, and monkeys. Although much valuable information has accrued from studies with these animals (see especially sections on Virulence and Chemotherapy), their disadvantages have led to a recent emphasis on mammals more convenient for mass experimentation, chiefly rats, but also guinea pigs, rabbits, and hamsters.

Jones (77, 78) undertook to standardize experimental infections in rats. He obtained regular, intense infections in weanling rats inoculated intracecally. Susceptibility diminished sharply as body weights increased above 30 gm. American workers have had similar results with heavier rats of corresponding ages (151), and the general methods of Jones have been widely exploited. Jones (77, 78) and Neal (111, 112) have described the course of infection in some detail. The severity of ulceration was maximal at four days, declining gradually thereafter. Spontaneous cures were the rule, total durations of parasitism ranging from 8 to 95 weeks. Cysts were found only sporadically. A normal commensal of rats, *E. muris*, which complicates the study of *E. histolytica*, has received considerable study by Neal (110).

Tobie (153) obtained fairly consistent, severe infections in eight to nine week old rabbits inoculated intracecally with trophozoites. With cysts administered orally to older animals infection was less regular, as in the experiments of previous workers. Carrera & Faust (26) infected guinea pigs with considerable uniformity by inoculation of trophozoites into the ileum. These animals have been the object of extensive investigations, particularly with respect to chemotherapy and the effects of diet (27, 132, 148, 149, 150). Hamsters were employed by Reinertson & Thompson (152) for chemotherapeutic studies, exhibiting regular, severe abscesses after intrahepatic injection of trophozoites. The only successful experiments on infection of non-mammalian hosts with *E. histolytica* appear to be those of Miller (102), who reported transient infections in young chicks.

VIRULENCE

It has long been clear that *E. histolytica* plays a part in the pathogenesis of disease of the intestine and other organs. This is evident both from extensive experimentation and from the specific histopathology of lesions associated with the amebae (see 38). The nature of the role played by the amebae is, however, not at all obvious. The prevalence of asymptomatic amebiasis and the apparently capricious relationship between amebic infection and disease bring the conviction that other factors are at least as important as the amebae in determining virulence. Some observers have even suspected that *E. histolytica* merely imposes a unique pathological character on lesions actually produced by bacteria. While no simple answers are at hand, much light has been cast on the problem by studies of diet of the host, of the participation of bacteria in initiation of "amebic" lesions, of strain differences among amebae, and of possible commensal habits of *E. histolytica*.

Influence of diet of the host.—The profound influence of diet of the host on the incidence and character of amebiasis has been clear since the work of Kagy & Faust (79) and Faust *et al.* (51). They showed that dogs, which normally exhibit severe diarrhea, achieved a chronic condition with production of cysts when fed on raw liver but experienced fulminating dysentery on a ration of canned salmon. It has been shown that virulence of the infection is enhanced by certain specific dietary deficiencies, notably nicotinic acid in dogs [Larsh (89)] and ascorbic acid in guinea pigs [Sadun *et al.* (132)]. The effect of a variety of diets on infection of rats and guinea pigs has been clearly demonstrated by Taylor *et al.* (150), but the significance of particular nutritional factors is not evident from the data.

Clinical findings are suggestive. Thus Alexander & Meleney (1) observed a greater prevalence of acute infections in populations on a substandard diet than in those more adequately nourished, although the incidence of infection was essentially the same. Similarly, Elsdon-Dew (49) has associated exceptionally severe amebic dysentery in African populations with a maize diet of poor quality. With some exceptions (27, 51, 150), the bulk of published clinical and experimental data tends to support the conclusion that a ration rich in protein minimizes the severity of amebiasis, while one high in carbohydrate enhances it. The next section will make it clear that this dietary effect may well be a result of modification of the intestinal flora associated with *E. histolytica*.

Influence of associated bacteria.—The well known fact that a much higher proportion of infected persons exhibits dysentery in the tropics than in temperate regions suggests that accompanying bacteria may contribute to the disease process (157). Andrew (157), for example, noted that the amebic dysentery rate among Australian troops was high in New Guinea, where bacillary dysentery was prevalent, and low in Australia, where there was little bacillary dysentery, although the incidence of *E. histolytica* was essentially the same in both areas.

Attempts to isolate specific bacteria from cases of amebic dysentery have produced merely suggestive results. Thus, Stewart (157) found paracolon bacteria, *S. faecalis* and *A. aerogenes* more common in acute cases than in controls, but it must be admitted that this may be a result of the altered intestinal conditions rather than a contributing cause of the disease.

Persuasive circumstantial evidence derives from the results of antibacterial treatment in amebic dysentery. Hargreaves (61) and others (157) have recorded marked clinical improvement, even some apparent cures, with antibiotics demonstrably inactive against the amebae themselves. This points strongly to a major influence of the bacteria on disease production.

The best evidence of bacterial influences in pathogenesis of "amebic" lesions comes from experimental data. Cleveland & Sanders (35) studied the induction of abscesses in cats inoculated intrahepatically with cultures of *E. histolytica*. Some strains were much more virulent than others. Exchange of bacteria between strains disclosed that the primary differences in pathogenicity were associated with the bacteria. Deschiens (43) and Nauss & Rappaport (109) have shown that certain bacteria, bacterial bodies, or chemical irritants augmented the proportion of severe intestinal infections in experimentally inoculated cats and rats. Westphal (158) obtained striking clinical evidence of the same effect in a small but crucial experiment. A self-induced infection with cysts of *E. histolytica* had remained evident but asymptomatic for several months. Ingestion of bacteria from a case of acute amebiasis was followed by a flare-up of typical amebic dysentery.

The data reviewed leave little room for doubt that, in both experimental animals and man, bacteria participate significantly in the production of "amebic" lesions. We must, indeed, seriously question whether *E. histolytica* is capable of tissue invasion by itself or may, in fact, require preparatory injury by other agents. While much more information is needed, the available facts at least contribute to an explanation of some of the apparent inconsistencies in the relationship of *E. histolytica* to disease.

Strain differences.—It is natural that one of the first explanations offered for the infrequency and unpredictability of disease in individuals infected with *E. histolytica* was the possible existences of races, varieties or types of amebae differing in virulence. Brumpt (23) went so far as to describe a distinct species, *E. dispar*, differing from *E. histolytica* only in being nonpathogenic. The hypothesis is attractive, but it has the serious defect that there is no practical way of determining to which of the two postulated species an ameba belongs.

It is possible, however, to test the pathogenicity of a strain of amebae. Frye & Shaffer (53) and Meleney & Frye (98, 99), with laborious and precise studies, determined the "pathogenic index" of various strains of *E. histolytica* in kittens which had been infected by inoculation of trophozoites into the lower ileum. They showed that strains from various sources differed in virulence, that the virulence of a strain remained generally constant for long periods, and that interchange of bacterial associates between strains did not

materially alter their pathogenic indices. Particularly noteworthy were four strains from Chicago, following the epidemic of 1933, which exhibited the highest indices of any strains tested, and a small cyst-producing strain, which consistently gave the lowest index. Similar data, although less extensive, have been recorded by Neal (112) from studies in rats. There appears to be a conflict between these data and those discussed above concerning the role of bacteria in the pathogenesis of "amebic" lesions, but the apparent disagreement is not necessarily meaningful. We may logically conclude that strains of *E. histolytica* differ in intrinsic virulence even if the expression of this innate virulence requires accessory bacterial factors.

Sapero *et al.* (134) brought together and clarified a wealth of evidence that strains of *E. histolytica* fall into one or the other of two distinct groups characterized by differences in cyst size. They pointed out that there was insignificant overlapping in size between strains which produced cysts less than 10 μ in diameter (nine μ in fixed material) and those with cysts larger than 10 μ. The small race, for which the names *E. hartmanni* and *E. histolytica hartmanni* have been suggested, differs also in motility, cultivability, and pathogenicity from the type form. Despite sporadic contrary claims (38), it appears that the small race is virtually nonpathogenic.

The observation of Meleney & Zuckerman (100) that a small cyst-producing strain acquired larger size (of trophozoites) during cultivation in new media elicited the suggestion that the difference was environmental. This explanation is discounted, however, by the fact that the two types of cysts often have been found together (134). Until *E. histolytica* is better understood genetically, the apparent conversion of one type to another should not suffice to deny the distinctness of the types. It must be conceded that the data point to the existence of a small cyst type of minimal pathogenicity and of a considerable variation in pathogenicity among the large cyst types.

Commensalism in Entamoeba histolytica.—The question whether *E. histolytica* lives only in the tissues or may also exist commensally in the intestinal lumen is not clearly settled. Craig (38) and others have maintained that in the human intestine the amebae are strictly tissue parasites. Any clear evidence that *E. histolytica* ever lives as a commensal in the lumen of the human intestine would disprove this contention. Hoare (64) seems to have found such evidence in the existence of ingested bacteria in 30 to 100 per cent of the amebic trophozoites from a number of chronic cases and carriers. Unfortunately, the basis for selection of cases in this study was not given. Further, there are some grounds for the counter-argument that the amebae examined were not in the colon but in passed stools. However, the supporting evidence marshalled by Hoare in a review of the question (65) is impressive. That, at least under some circumstances, *E. histolytica* can multiply outside the tissues is proven by artificial cultivation and particularly by the nature of infections in macaque monkeys, which harbor *E. histolytica* as an indubitable commensal. While the debate has rested mostly on testimony, and we might wish for more data, it may tentatively be con-

cluded that *E. histolytica* often leads a nonparasitic existence in the lumen of man's intestine.

General conclusions regarding virulence.—Natural immunity has not been mentioned as a factor in the severity of infections with *E. histolytica*. The classical experiments of Walker & Sellards (156) intimated that natural resistance was responsible for the failure of some volunteers to exhibit dysentery with strains of amebae which caused disease in other persons. The preceding discussion should have made it plain that possible differences between individuals in both diet and intestinal floras forbid an answer to this question. Recognizing the hazard of oversimplification, we may summarize the evidence presented on virulence of *E. histolytica* with the generalizations that (*a*) the small cyst-producing type of *E. histolytica* is virtually nonpathogenic in man, (*b*) the large cyst type varies greatly in virulence, (*c*) in carriers the amebae may often live as commensals in the lumen of the intestine, and (*d*), at least with strains of moderate virulence, the initiation of significant lesions in the tissues requires both minimal natural immunity and accessory action by bacterial, dietary, or other facors.

ACQUIRED IMMUNITY

Acquired immunity has been held responsible for the decline in incidence of amebiasis beyond middle life. Actually, there seems to be no clear evidence on acquired immunity in man and very little in animals. The best indications are seen in the work of Swartzwelder & Avant (146), who reported that dogs which had recovered spontaneously or had been cured with drugs were markedly resistant to reinfection. Transfer of blood from immune animals seemed to confer some protection on recipients. The fact that experimentally infected animals generally recover spontaneously (e.g., see 111), at least from the acute phase of infection, suggests the acquisition of immunity, but the features of this recovery have not been analyzed. Furthermore, it should be realized that immunity to the associated bacteria can have an influence on recovery from amebic lesions. Partial protection of rats has been induced by artificial immunization with an alcoholic extract of *E. histolytica* [Swartzwelder & Muller (147)].

A variety of immunological reactions has been recorded in amebic infections or antigen-injected animals. The greatest attention has been directed at the complement-fixation reaction, first standardized by Craig. The main test antigens employed have been alcoholic extracts of poly- or monobacterial cultures, washed cysts, amebic lesions, or dysenteric stools (39, 126). Most recent studies have utilized a commercial antigen prepared essentially by the method of Rees *et al.* (126) from flask cultures of *E. histolytica* grown with "organism t." Control tests with antigens from the bacterial associate have shown only insignificant reactions.

As a method for routine laboratory diagnosis of amebiasis, complement-fixation has few modern supporters. The major current interest in complement-fixation lies in its assistance in diagnosis of amebic hepatitis. Here,

with a relatively insensitive test, using a short fixation period, Hussey & Brown (69) and others have obtained a high proportion (83.4 per cent) of positive reactions in hepatic amebiasis, with most cases of purely intestinal infection (97.6 per cent) reacting negatively.

The paucity of research on other immune phenomena is surprising, unless one recognizes the difficulty of distinguishing purely antiamebic reactions when the antigens invariably contain bacterial substances. Apparently specific skin reactions have been reported by Menendez (101) in rabbits. Menendez also described lysins, precipitins, and immobilizing antibodies, and precipitins of high titer were obtained by Heathman (63). Greif (57) observed agglutination of cysts in human sera, but there was no discernible relationship between this phenomenon and the presence of amebic infection.

CHEMOTHERAPY

The search for amebacidal drugs is one field which has been scarcely affected by inadequacy of technical methods. Since the burgeoning literature cannot be covered comprehensively, only the main trends will be examined, and the discussion will be limited to those drugs which have had extensive clinical study or which show exceptional specific activity in experimental work.

Older testing methods utilized primarily experimental infections in dogs or monkeys or natural amebiasis in monkeys. While these systems are still useful (3), recent emphasis has shifted to smaller mammals and to *in vitro* studies. Experimental amebiasis in rats has received the most extensive study. Goodwin *et al.* (56), Jones (77, 78), and Thompson *et al.* (151) have developed efficient standard procedures for testing of drugs in intracecally inoculated young rats. They have observed excellent correspondence between the test results and the clinical effectiveness of recognized amebacides, and have evaluated extensive series of potentially valuable compounds. Rabbits (91) and guinea pigs (149) have also shown preliminary indications of value as test animals. Experimental infections in kittens are of doubtful utility, for such infections have been shown to be unresponsive to emetine and variable in their susceptibility to other drugs (32). Thompson & Reinertson (152) have found experimental hepatitis in young hamsters a convenient tool for chemotherapeutic studies, revealing activity in compounds effective against human amebic hepatitis, such as chloroquine and emetine, but not in those used only for intestinal infection.

Cultures of *E. histolytica* have obvious value in drug studies and have been employed extensively both for screening tests and for investigation of modes of drug action. Two problems arise with their use. First, drugs which are metabolized into active compounds *in vivo* may fail to show *in vitro* activity. Second, in the complex ecology of amebic cultures apparent action of drugs may result from indirect effects, such as destruction of the microbial associates of the amebae. Bradin & Hansen (19), noting that several antibiotics were markedly less active in anaerobically sealed cultures than in

cotton-plugged tubes, concluded that some or all of their alleged amebacidal action was a result of inhibition of the associated bacteria (see also 60). That antibiotics may destroy *E. histolytica* directly has been shown by Balamuth & Brent (11) with prodigiosin, which was inactive against the bacterial associates, by Bradner & Rawson (21) and others (106, 107, 117) in cultures with *T. cruzi*, which was unaffected by the drugs, and by Shaffer & Biegeleisen (138) in cultures of amebae with nonreproducing bacteria.

Evaluation of the clinical effectiveness of amebacides is particularly complex. The laboratory diagnosis of amebiasis is difficult at best, and the reduction in numbers of amebae which follows partially successful treatment increases the likelihood of failure in finding parasites. Assessment of cure requires several stool examinations, 10 or more according to most careful workers, over a period of several months. Apparent relapses may occur as long as a year after treatment. Differences in the efficiency of diagnosis in different laboratories obviously can complicate a comparison of drugs tested by different workers. Finally, the clinical character of cases may affect the relative success of a particular therapy. An extreme example of the variation in different clinical reports is seen in the case of *p*-ureidobenzenearsonic acid (Carbarsone). In a series of peculiarly violent cases of dysentery, Armstrong *et al.* (7) achieved clearance of amebae in less than 50 per cent, while in a group of carriers and chronic cases Anderson & Reed (6) cured over 90 per cent.

Emetine, an alkaloid of Ipecacuanha (*Cephaelis ipecacuanha*), boasts a long history of use in the therapy of amebiasis. It kills *E. histolytica in vitro* at extremely low dosage, 1 p.p.m. or less (10, 45). The action of emetine, unlike that of most other amebacides, is very specific, *E. coli* and other intestinal amebae requiring approximately 100 times the concentration necessary to destroy *E. histolytica* (10). There have been claims that amebae became resistant to emetine, but these reports have not been verified, and the apparent effect has been attributed to uneven distribution of drug in diphasic culture media (45). Despite its intense amebacidal action and the fact that it usually leads to prompt clinical improvement, emetine rarely eliminates amebic infection (38). This defect, together with its serious toxicity, has brought about a general clinical replacement of emetine by more efficiently curative and less toxic drugs. Extensive studies of compounds chemically related to emetine have failed to reveal agents of significant value (55).

Preparations of Kurchi bark (*Holarrhena* spp.) have long been employed in treatment of dysenteries, and the major alkaloid, conessine, destroys *E. histolytica* rapidly *in vitro* (38). Durieux *et al.* (48) reinvestigated the antiamebic properties of conessine and reported a high rate of clinical cures, often in cases which had failed to respond to emetine. Their studies have aroused marked interest in conessine, particularly among French workers, but most investigators consider its serious toxicity, particularly its neuropsychiatric effects, an important obstacle to general clinical use (24).

Several 7-iodo-8-hydroxyquinolines have been widely used in amebiasis. The major representatives of this class are 7-iodo-8-hydroxyquinoline-5-sul-

fonic acid (chiniofon), 5-chloro-7-iodo-8-hydroxyquinoline (vioform), and 5,7-diiodo-8-hydroxyquinoline (diodoquin). These compounds have given cure rates of over 90 per cent, although some studies have indicated 20 per cent or more uncured cases (7, 38).

Three groups of arsenicals are prominent in the treatment of amebiasis. Carbarsone has produced cures in as high as 90 per cent of carriers and chronic cases [Anderson & Reed (6)]. It has sometimes proven much less effective, particularly in acute dysentery (7). Carbarsone oxide was reported considerably more active than carbarsone in experimental animals (4). The equally active but less toxic dithiocarboxyphenyl and dithiocarboxymethyl derivatives of carbarsone oxide, tested by Anderson *et al.* in 40 patients each, cured 95 per cent (5). Bismuthoxy-*p*-N-glycolylarsanilate (Milibis) has produced reported cures in about 90 per cent of cases (16). Milibis is said to exhibit extremely low toxicity and has been recommended as a prophylactic agent for temporary visitors to the tropics (41).

Since the report of McVay, Laird & Sprunt (97) that amebic dysentery could be cured by aureomycin, an imposing literature has accumulated concerning the use of antibiotics in amebiasis. The most important are aureomycin, terramycin, and fumagillin, but a number of others has shown experimental or partial clinical effectiveness, notably prodigiosin, simarubidin, actidione ($C_{15}H_{23}NO_4$) and bacitracin (2, 10, 11, 40, 104).

Aureomycin is effective in all types of intestinal amebiasis and produces especially prompt clinical improvement (97). *In vitro* it exhibits slight but definite amebacidal action (10, 97), and it appears that the clinical effectiveness is a result of both antiamebic and antibacterial properties. Despite its rapid action, it probably does not cure more than 80 per cent of cases (52). Terramycin appears somewhat more effective than aureomycin in human amebiasis, although its *in vitro* activity against *E. histolytica* is comparable (10). Cures have been reported in 85 to 97 per cent of cases [Most & Van Assendelft (103) and Frye *et al* (52)].

Fumagillin has shown the highest *in vitro* activity reported for any amebacide, lethal concentrations ranging from 1:10,000,000 to 1:100,000,000 [McCowen *et al.* (95); Hrenoff & Nakamura (68)]. Unlike the other antibiotics discussed, it is essentially inert toward bacteria. In preliminary clinical studies it has exhibited therapeutic efficiency of the order of terramycin in chronic cases, but doubtful effectiveness in acute cases [Killough *et al.* (85)]. Fumagillin must be considered a most promising agent for therapy of amebiasis, but conclusive assessment of its value is impossible at present.

As far as is known none of the drugs discussed above has therapeutic value in extraintestinal amebiasis except emetine, which was, until recently, the drug of choice for such conditions. In 1948 Conan (36) demonstrated that chloroquine was highly effective in amebic hepatitis. This finding has been abundantly confirmed, and the related drug, Camoquin [7-chloro-4-(3-diethylaminomethyl-4-hydroxyanilino) quinoline] has shown similar activity (66). Chloroquine has also exhibited clinical value in extensive pleural amebiasis associated with hepatic infection (37). Despite its significant amebacidal

action *in vitro*, chloroquine appears useless in intestinal amebiasis, presumably because it is largely absorbed in the upper intestine.

LABORATORY DIAGNOSIS

Present knowledge of laboratory diagnosis in amebiasis is grossly unsatisfactory. The best methods available depend ultimately on subtle morphological recognition of *E. histolytica*. This is difficult for experts and dangerously inaccurate in the hands of many inadequately trained technicians.

Basically, laboratory diagnosis of amebiasis rests on microscopic identification of *E. histolytica* in fresh saline smears of feces or in stained preparations. Until recently iron hematoxylin was the preferred stain for trophozoites and hematoxylin or iodine-potassium iodide for the cysts. These methods have been thoroughly reviewed by Craig (39) and do not require consideration here. Certain supravital staining methods which have recently come into wide use should, however, be mentioned. The methylene blue-Sudan III method of Quensel (see 39) gives excellent differentiation of trophozoites, as does the crystal violet-triethanolamine-hematoxylin method of Velat, Weinstein & Otto (155). Good staining of both cysts and trophozoites is obtainable with the merthiolate-iodine-formalin technique of Sapero, Lawless & Strome (135). While these procedures are not intended for the preparation of permanent specimens, they have the considerable advantage over iron hematoxylin that they are simple and rapid and that they give adequate cytological differentiation for diagnostic purposes.

Two accessory procedures are useful in diagnosis. The polyvinyl alcohol technique of Goldman (54) permits storage and shipping of fecal specimens, which can be stained with iron hematoxylin and examined by competent technicians in a central laboratory. The formalin-ether concentration method of Ritchie (129) is said to reveal many infections missed by the zinc sulfate flotation method (see 39). If further studies support these claims, this technique will be the most sensitive diagnostic method available for cysts of *E. histolytica*.

Recent studies have endeavored to determine the absolute efficiency of diagnostic methods (93, 154). Mantel's method (93) is ingenious, particularly since it permits evaluation of a technique without the need for comparison with another technique. Adequate emphasis has not been given, however, to the oversimplification involved in its basic assumption, namely, that all infections have equal "demonstrability" (88). Intensity of infection, and therefore demonstrability, varies widely from person to person and from time to time in the same person. The probable existence of infections of such low intensity that they are almost certain to be missed by any diagnostic method suggests that the "true efficiencies" reported are too high and the "true prevalences" too low (154).

Despite sporadic claims to the contrary, most investigators continue to find culture methods unsatisfactory for diagnosis. The presence in stools of inimical bacteria appears to interfere with cultivation of *E. histolytica* in

many cases. While antibiotic treatment has increased the proportion of positive cultures materially, microscopically positive stools frequently produce negative cultures (143).

For the important role of complement-fixation in diagnosis of extraintestinal amebiasis, see pp. 284–285.

EPIDEMIOLOGY AND CONTROL

The chief possible mechanisms for transmission of amebiasis are well known (38). They are food, water, fomites, flies, and direct contact. While the usual sources of endemic spread have not been at all clearly defined, each of the mechanisms listed has been circumstantially incriminated in one or more specific situations.

Water-borne cysts were blamed for the notorious Chicago hotel epidemic of 1933, and the evidence, although indirect, was convincing (96). Local contamination of a water supply has been suspected also of responsibility for extensive infection in a Tokyo apartment house (130).

Craig (38) presented good evidence that domestic flies were important vectors in an army epidemic. Westphal (159) found unusual abundance of flies associated with a high incidence of amebiasis in North Africa and observed amebic cysts in wild-caught flies. Harris & Down (62) cultivated *E. histolytica* from feces and vomitus of flies captured near areas on Guam where human infection was prevalent. These clear epidemiological proofs, reinforced by the experimental data of Pipkin (119) and Roberts (131), leave scant room for doubt that domestic flies can contribute to the spread of amebiasis.

The importance of fomites and direct contact was emphasized by Ivanhoe (71), who related gross fecal contamination of clothes, bedding, etc. to the very high incidence of amebic infection in an orphanage. The considerable susceptibility of cysts of *E. histolytica* to drying [Reardon *et al.* (123)] suggests that fomites are insignificant in most circumstances. That soil contamination may play a part in transmission is indicated by the demonstration of Beaver & Deschamps (14) that cysts could survive as long as eight days in soil samples.

The significance of spread by food handlers is difficult to evaluate. Older experiments indicated poor survival of cysts on workers' hands, and some epidemiological studies have minimized the role of food-handlers. Schoenleber (136), however, has produced very convincing evidence that they may constitute a major hazard. In a foreign colony in Venezuela, under circumstances which seemed to rule out other means of transmission, he obtained excellent control of amebiasis with procedures directed solely at reducing the incidence of infection in food handlers and improving their hygienic practices. The likelihood of familial spread, particulary in domestic food preparation, has recently been accented by D'Antoni (41). Other mechanisms of contamination of food seem not to have been clearly incriminated, although the danger of rinsing fruits and vegetables with polluted water must not be ignored.

With the exception of Schoenleber's work (see above), data are lacking on the actual results of efforts directed specifically toward control of amebiasis. Nonetheless, there is considerable information on the experimental effectiveness of various procedures in destruction or elimination of cysts. Older studies on the action of chlorine gave very misleading results, primarily because of failure to allow for the interference by organic matter in the test suspensions and to base conclusions on accurately determined effective chlorine concentrations (38). More recent studies by Chang (28) and Newton (114) have shown that chlorine residuals of the order of 1 to 2 p.p.m. rendered amebic cysts nonviable. These concentrations are somewhat greater than are required for destruction of enteric bacteria and are not attained in ordinary water treatment practice. Superchlorination, however, can produce amebacidal levels. Certain detergents destroy amebic cysts, cationic types exhibiting particular activity (50, 84). Because of its potential value in sterilization of fresh vegetables, acetic acid has received some attention. Beaver & Deschamps (15) reported destruction of cysts in water with a 10 to 15 min. exposure to 5 per cent solutions. Jones (75) observed that while this concentration achieved complete killing of cysts in water it did not sterilize all cysts on experimentally contaminated vegetables. Heat has a marked effect, and temperatures above 52°C. have been reported to be cysticidal in less than one minute (76).

In the face of abundant evidence (38, 157, 160) that amebiasis is widespread in the general population, it may seem surprising that public health agencies have evinced only minor concern with it. The key to this question seems to lie in the unsolved problems of virulence of *E. histolytica*. The meaning of the figures obtained in surveys of prevalence is not at all clear. In those studies where the distinction was made, it has been reported that between one-third and two-thirds of all infections exhibited the small cyst-producing type of amebae (65, 134). This fact, together with the known complexity of the pathogenesis of amebic dysentery, casts serious doubt on the epidemiological import of data on the incidence of *E. histolytica*. An accurate evaluation of the significance of *E. histolytica* in public health will be possible only with elucidation of the real part played by the parasite in production of disease.

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HELMINTHS: METABOLISM, NUTRITION, AND CHEMOTHERAPY[1,2]

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METABOLISM

Many metabolic reactions in helminths are similar to those occurring in higher and lower forms of life. However, marked biochemical differences have been observed also. As yet, our knowledge of this field is too limited to provide even a tentative answer to the problem of whether these differences can be ascribed to a process of biochemical adaptation to the habitat and the mode of life of the parasite.

This section deals mainly with investigations published during the past 4 years. Earlier work has been reviewed previously (1). The reader is referred also to Von Brand's recent book on chemical physiology of parasites (2).

Carbohydrate metabolism.—For a long time it has been known that helminths have a high rate of carbohydrate metabolism, but virtually no information has been available concerning the intermediate reactions and pathways of carbohydrate utilization. Recent work has supplied evidence for the occurrence of the Embden-Meyerhof scheme of phosphorylating glycolysis; this was observed in every helminth in which this problem has been studied. Rogers & Lazarus (3) have reported an increase in acid-soluble organic phosphate and a reduction in inorganic phosphate during incubation of extracts of *Ascaris lumbricoides*. These changes are more pronounced in the presence of fluoride which inhibits the activity of phosphatases. Fructose-1,6-diphosphate is utilized by extracts of two other nematodes, *Nematodirus sp.* and *Ascardia galli;* the latter organism contains phosphorylase activity (3).

The presence of aldolase, triosephosphate dehydrogenase and of lactic dehydrogenase has been demonstrated in extracts of *Ascaris* muscle (4). Lactic acid is formed by these extracts in the presence of glucose, of ATP,[2] and of magnesium (4). Lactic acid production is increased when fructose-1,6-diphosphate is used as substrate and when inorganic phosphate is replaced by arsenate. Addition of DPN[2] has no effect on lactic acid production by these extracts, even after they have been dialyzed thoroughly. Apparently,

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[1] The survey of literature pertaining to this review was concluded in February, 1953.

[2] The following abbreviations are used in this chapter: ATP (adenosinetriphosphate); DPN (diphosphopyridine nucleotide); AMC (acetylmethylcarbinol).

DPN is bound to the triosephosphate dehydrogenase of *Ascaris* muscle more strongly than to that of mammalian skeletal muscle (5). Lactic acid also may be produced in *Ascaris* from methylglyoxal because of the presence of a glyoxalase (6). However, the physiological significance of this enzyme, which is distributed widely among many species of animals (6), has remained obscure.

Observations suggesting the presence of several enzymes concerned with phosphorylating glycolysis in the cestode *Hymenolepsis diminuta* have been reported by Read (7). In addition, the production of lactic acid and esterification of inorganic phosphate have been demonstrated in homogenates of this tapeworm (8). In a fractionated extract from this helminth triosephosphate dehydrogenase activity was detected in the absence of added DPN. Apparently, as in the case of *Ascaris*, this coenzyme is bound firmly to the dehydrogenase of *H. diminuta*.

It is of interest that no "phosphagen" (arginine or creatine phosphate) could be found in *Ascaris* (3) nor in *H. diminuta* (7). The absence of phosphagen in these two helminths might suggest that a parasitic mode of life does not require the storage of an energy-rich phosphate reserve capable of rapidly regenerating ATP.

Utilization of glucose proceeds at an extremely high rate in the trematode *Schistosoma mansoni* (9). The parasite metabolizes in 1 hr. an amount of glucose equivalent to approximately one-fifth of its dry weight. At least 80 per cent of the carbohydrate metabolized can be accounted for as lactic acid (9). Incubation of homogenates of schistosomes with glucose results in a rate of formation of lactic acid which is at least as high as that of intact worms of equal weight (10). In addition to glutathione and Mg, ATP and DPN are required for lactic acid production from glucose by schistosome extracts. Lactic acid production is greater when fructose-1,6-phosphate is used instead of glucose. The presence of the following glycolytic enzymes in extracts of schistosomes has been demonstrated: hexokinase, isomerase, aldolase, triosephosphate dehydrogenase, and lactic dehydrogenase (10). In addition to these enzymes the schistosomes contain two adenosinetriphosphatases; one of them requires magnesium ions, the other calcium ions for optimal activity (10).

Two types of enzymes are known which catalyze the phosphorylation of hexoses by ATP. Yeast and mammalian tissues contain a hexokinase reacting with glucose, mannose, and fructose (11, 12, 13, 14). In addition, a specific fructokinase is found in liver and muscle (14, 15); this enzyme does not act on other hexoses. In male schistosomes several hexokinases are present. One of them is specific for glucose (16). However, in the presence of ATP, homogenates and extracts of male schistosomes also phosphorylate mannose and fructose (16). Several observations indicate that phosphorylation of these two hexoses is catalyzed by two different enzymes. In contrast to male schistosomes, homogenates of some female worms exclusively phosphorylate glucose, but do not react with mannose or fructose (16). Possibly the occur-

rence of hexokinases which do not react with mannose is not limited to schistosomes. Cavier & Savel (17) have observed that, in contrast to glucose and fructose, mannose is not utilized by *Ascaris* for glycogen synthesis. This may be caused by the inability of *Ascaris* tissues to phosphorylate mannose, although the absence of phosphomannose isomerase (18) from the tissues of this parasite should be considered also as an alternative explanation. It has been pointed out previously (19) that the nature of enzymes which catalyze the same reaction in the parasite and in the host may not necessarily be identical. This hypothesis is supported by the observed differences in the properties of the hexokinases of schistosomes and of mammalian tissues. Similarly, differences between the lactic dehydrogenase of rabbit muscle and that of *S. mansoni* have been observed. For example, the optimal activity of the parasite's enzyme extends over a much wider pH range. Furthermore, sera from chickens which have been immunized against the lactic dehydrogenase of rabbit muscle inhibit the activity of this enzyme to an extent of 50 to 78 per cent. On the other hand, these sera do not affect the activity of lactic dehydrogenase of *S. mansoni* (20). These findings indicate that the two enzymes are not identical.

According to Neuberg (21), the production of acetylmethylcarbinol is an omnicellular process, since it occurs in various plants, yeast, bacteria, and mammalian tissues. This view is supported by the occurrence of several AMC[2] producing systems in the filarial worm *Litomosoides carinii* (22). Only this organism and *Aerobacter aerogenes* produce AMC in the presence of glucose, their physiological substrate. Pyruvate, acetaldehyde, or both, usually in fairly high concentrations, are required for the production of AMC by other biological systems. It should be noted that the mechanisms of AMC formation in the filariae are different from those in *A. aerogenes*; in the latter α-acetolactate is an intermediate, and acetaldehyde is not involved in this reaction (23); on the other hand, α-acetolactate has been ruled out as an intermediate in the formation of AMC by the filarial systems which utilize acetaldehyde for this synthesis (22). Production of AMC from acetaldehyde by cell-free extracts of these worms requires cocarboxylase and manganese and is stimulated greatly by pyruvate. Differences in the effects of pH, of magnesium, and of ageing of the filarial preparations indicate that AMC production from acetaldehyde alone on the one hand, and from acetaldehyde and pyruvate on the other, is catalyzed by two different enzymes (22). AMC is utilized by the filariae aerobically and, at a considerably slower rate, anaerobically. The ($-$) isomer which is formed by the worms is utilized much more rapidly than is ($+$)-AMC (22). It is of interest that incubation of the cestode *Raillietina cesticillus* with the anthelmintic drug hexachlorophene (24) results in the accumulation of a compound which gives a positive Voges-Proskauer test (25), and that low concentrations of hexachlorophene inhibit the utilization of AMC by *L. carinii* (22). The inhibitory effect of hexachlorophene on the utilization of AMC suggests the possibility that this process is an essential metabolic reaction in the filariae.

Under anaerobic conditions extracts of *Nematodirus* sp. and of *A. galli* remove carbon dioxide (3); this observation indicates the presence of a mechanism of CO_2-fixation. According to observations of Glocklin & Fairbairn (26), carbon dioxide exerts a carbohydrate sparing effect in *Heterakis gallinae*. In this nematode Fairbairn (27) has observed a mechanism of carbon dioxide fixation, without any net assimilation of carbon dioxide, similar to the reactions occurring in propionic acid bacteria during the fermentation of glucose (28).

Evidence for the occurrence of a number of carbohydrases in helminths has been reported. Rogers (29) has observed marked differences between the properties (e.g., pH optima, activation by various ions) of intestinal amylases of *A. lumbricoides* and those of *Strongylus edentatus*. These observations supply another example of the biochemical differences existing among morphologically related helminths. The perienteric fluid of *Ascaris* contains saccharase and maltase, but no lactase (30). This is corroborated by the ability of intact *Ascaris* to synthesize glycogen from sucrose and maltose, while no glycogen synthesis occurs in the presence of lactose (17). The reports of Levine *et al.* (31) and of Stirewalt & Evans (32, 32a) about the hyaluronidase activity of cercariae of *S. mansoni* might explain, in part, the mechanism by which these organisms penetrate the skin. It would be of interest to determine whether the activity of this enzyme, in conjunction with those of proteolytic and collagen-splitting enzymes, can account for the rapid entrance of the cercariae into the mammalian host. It would appear improbable that penetration through the outer (keratin) layer of the skin could be achieved or facilitated by the action of hyaluronidase.

Lipid metabolism.—The question whether helminths can utilize lipids or fatty acids has been the subject of some investigations. Von Brand (33) has demonstrated that the small decrease in the total lipids occurring during starvation of *A. lumbricoides* can be accounted for completely by the fat transferred to the excreted eggs. Therefore, in this study no evidence for the utilization of lipids could be obtained. However, at the end of the experimental period of five days, the glycogen stores of the worms had not been depleted; perhaps some utilization of fat might occur after longer periods of starvation.

Lipase activity has been demonstrated in the perienteric fluid of *Ascaris* by Cavier (17) and, with the aid of histochemical methods, in the subcuticula of some acanthocephala by Bullock (34). The presence of lipases in the intestinal tracts of *A. lumbricoides* and of *S. edentatus* has been detected by Rogers (35). The ability to digest fat does not necessarily indicate utilization of fatty acids. Possibly only the alcohol moiety (e.g., glycerol) is metabolized, while the fatty acids are excreted. Pflugfelder (36) observed fat absorption and storage in *Acanthocephalus ranae*, but he reported no experimental evidence for fat utilization.

Recent observations of Von Brand *et al.* (37) on the metabolism of bacteria-free *Trichinella* larvae have supplied the first unequivocal demonstra-

tion of lipid utilization by a parasitic worm. These authors observed a marked reduction in the total fat content of the larvae under aerobic conditions. Assuming complete oxidation of the lipids to CO_2 and water, more than 50 per cent of the oxygen uptake of the worms could be accounted for by this process, which may function as a major source of energy for the larvae. It is of interest that fat oxidation occurs in this helminth concurrently with glycogen consumption.

Jennings (38) has extracted from *Haemonchus contortus* the antigen concerned in the complement fixation reaction with natural antisera from sheep infected with this helminth. This material has the properties of an acidic hydrophylic lipid. It is unstable to oxidation, and its molecular weight appears to be above 1000. Presumably it is bound in the tissues of the worm with a protein. The antigen is present in the eggs, the third stage larvae, and the adults of *H. contortus*, as well as in a number of other helminths, such as the adults of *Nematodirus*, of *Moniezia*, and of *Ascaris*, as well as in *Strongyloides* larvae, but it is absent in *Fasciola hepatica* and in vertebrate tissues. It would be of interest to determine the significance of this lipid as a constituent or as a metabolic product in those helminths in which its presence has been ascertained. A protein-bound lipid with hemolytic properties has been found in *Ascaris* by Masquelier & Bailenger (39).

In addition to various lipases, acetylcholine esterase occurs in some helminths. *S. mansoni* contains an enzyme the concentration and many properties of which are similar to those of the specific acetylcholine esterases of mammalian brain (40). The only observed difference between these two enzymes consists in a higher Michaelis constant of acetylcholine for the enzyme from schistosomes. High activities for acetylcholine esterase have been observed also in the filarial nematode *L. carinii* (40) and in the trematode *F. hepatica* (41). Cell-free preparations of the latter helminth (41) and of *S. mansoni* (42) contain choline acetylase, i.e., an enzyme which catalyzes the synthesis of acetylcholine from choline and acetate in the presence of ATP and coenzyme A (43). Chance & Mansour (41, 44) have made the interesting observation that physostigmine and carbaminoylcholine have a paralyzing effect on *F. hepatica* and on a deganglionized preparation of this trematode, while acetylcholine has no such effect. However, after incubation of a deganglionized preparation with physostigmine, acetylcholine produces a reduction or disappearance of motor activity. These effects are antagonized by atropine. Amphetamine and other sympathomimetic amines stimulate motor activity. Therefore, adrenergic drugs stimulate and cholinergic drugs depress the muscular activity of *Fasciola*. The presence of a potent acetylcholine esterase in this helminth explains why acetylcholine is effective only after treatment of the preparation with physostigmine, a strong inhibitor of acetylcholine esterase. By contrast, physostigmine does not potentiate the stimulatory effect of acetylcholine on the muscle of *Ascaris* (45). This tissue has an exceedingly low acetylcholine esterase activity (40).

Production of volatile acids.—Interpretation of the classical observations

of Weinland (46) that pentanoic and hexanoic acids accumulate in media in which *Ascaris* are incubated aerobically or anaerobically has been the subject of considerable controversy. The formation of these acids by Metazoa is most uncommon. On the other hand, the habitat of *Ascaris*, the host's intestinal tract, harbors a wide variety of bacteria, some of which are known to produce valeric and caproic acids (47, 48). Therefore, the occurrence of volatile fatty acids in *Ascaris* media has been ascribed either to the metabolic activities of contaminating bacteria, to those of *Ascaris* itself, or to combined biochemical reactions of helminths and of bacteria. The probability that *Ascaris per se* can produce pentanoic acids has been pointed out previously (1). However, more conclusive evidence bearing on this problem has been obtained by studies with bacteria-free *Ascaris*. These organisms can be obtained by the use of a mixture of antibiotics (49). While no single antibiotic removes all bacteria from the parasite, effective sterilization of a significant number of worms is obtained with combinations of penicillin, streptomycin and bacitracin (49), or terramycin (4). After incubation of the worms in these mixtures they were placed, for 24 to 48 hr., in antibiotic-free sterile media. The latter were analyzed for volatile acids, provided the absence of bacteria from the media and the worms had been ascertained by bacteriological culture techniques (49). It was found that in the presence of glucose bacteria-free *Ascaris* produce per 100 gm. (wet weight) in 24 hr. 2.5 to 4 m. eq. of volatile acids. Over 40 per cent of them have the solubility characteristics of acids containing five carbon atoms, 20 to 30 per cent behave as C_6- and 2 to 5 per cent as C_4-acids; 20 to 25 per cent consist of acetic and 10 per cent of propionic acid (4, 49). The C_5 fraction is a mixture of three acids. Isolation and identification of the components of this fraction revealed that bacteria-free *Ascaris* produce racemic α-methyl-butyric acid (4), *n*-valeric acid (50) and *cis*-α-methylcrotonic (tiglic) acid (51). It is not known whether other metazoa produce α-methylbutyric acid. The dextrorotatory isomer has been isolated from a number of plant sources (52, 53, 54) and has been identified as a product of certain microorganisms (55, 56, 57, 58). However, occurrence of racemic α-methylbutyric acid in nature hitherto has not been reported. Esters of tiglic acid are found in some plants (53, 58 to 62), but this acid has not been known as a constituent or product of animals or microorganisms. No definite information is available about the origin of these large quantities of volatile acids. They cannot be products of the metabolism of lipids; *Ascaris* does not utilize higher fatty acids during a period of starvation for at least five days (33). Yet, the volatile acids are produced during much shorter periods of incubation in the presence of glucose, a substrate which increases the formation of these acids. According to the analyses of Von Brand (63), the excretion by *Ascaris* of total nitrogen, including that contained in the eggs, is too low to account for the formation of volatile acids from degradation of proteins and amino acids. On the other hand, intact bacteria-free *Ascaris* produce only insignificant amounts of lactic acid although the presence of the enzyme systems involved in the conversion of

carbohydrate to lactic acid has been demonstrated in dialyzed cell-free extracts of this helminth (4). In view of these observations and also of the high rate of carbohydrate utilization of *Ascaris* (63), the possibility must be considered that the volatile acids produced by this helminth originate from carbohydrate, from lactic acid, or from one of the latter's precursors.

An analysis of the volatile acids of *Ascaris* contaminated with the bacterial flora of the pig's intestinal tract has been carried out by Moyle & Baldwin (64). They reported that these worms contain α-methylbutyric acid which exhibited a slight levorotation, equivalent to 6 per cent of the optical activity of the pure ($-$) isomer. Several bacteria are known to produce ($+$)-α-methylbutyric acid (56, 57), and it is possible that they also preferentially metabolize this isomer in the bacterial contaminated worms, while the helminths themselves produce racemic α-methylbutyric acid (4). No tiglic or n-valeric acid was found in *Ascaris* contaminated with bacteria (64); perhaps the latter metabolize these acids when they are produced by *Ascaris*. This is a distinct possibility because little or no tiglic acid is found in media in which bacterial contaminated *Ascaris* have been incubated (4). It is conceivable that the removal of tiglic acid is brought about through a reduction to α-methyl butyric acid by bacterial hydrogenases. However, the failure of Moyle & Baldwin (64) to detect several metabolic products of *Ascaris* may be ascribed also to qualitative and quantitative differences between the acids present in the worms and those produced by them and diffusing into their culture media. Therefore, it would appear that a more reliable picture of the chemical composition of *Ascaris* and of other intestinal helminths can be obtained if, henceforth, analyses are restricted to bacteria-free parasites.

Von Brand *et al.* (37) have observed that bacteria-free larvae of *Trichinella spiralis* produce volatile acids containing two to six carbon atoms; chromatographic analysis revealed that at least 50 per cent of them consisted of a C_5-acid which has the solubility characteristics of n-valeric acid. In contrast to *Ascaris*, *Trichinella* larvae produce no other pentanoic or pentenoic acids (37); no formic acid and only traces of lactic acid can be detected in the media in which the larvae have been incubated. Similar findings regarding these two acids have been made with *Ascaris* (4). It should be noted that both the glycogen consumption and the production of volatile acids by *Trichinella* larvae are the same under aerobic and anaerobic conditions (37). On the other hand, the lipid content is decreased only under aerobic, but remains constant under anaerobic conditions (37). Therefore, as in the case of *Ascaris*, the volatile acids may be products of carbohydrate metabolism but cannot originate from lipids.

Proteins and amino acids.—The presence in homogenates of *F. hepatica* of α-ketoglutaric transaminase activities with a number of amino acids has been demonstrated by Daugherty (65). Highest transaminase activity has been observed with aspartic acid, isoleucine, leucine, and valine, lower activity with arginine, alanine, phenylalanine, tyrosine, methionine, and pro-

line, while questionable or no transamination occurs in the presence of tryptophan, lysine, and cystine. Some transamination reactions are stimulated by pyridoxal phosphate. Similar transaminase systems have been found in *H. diminuta* and in *Macracanthorhyncus hirudinaceus* (66). Since the kinetics of these reactions have not been studied it is not possible to compare the properties of these enzyme systems in helminths with those present in mammalian tissues and in bacteria.

A most striking difference between the hemoglobins of vertebrates and those of intestinal nematodes consists in the extremely high affinity of the latter for oxygen. In addition, their affinity to carbon monoxide is lower. These properties have been demonstrated for the hemoglobins of the body wall and of the perienteric fluid of *Ascaris* (67), and for the hemoglobins of *Nippostrongylus muris* (68, 69), *Strongylus* sp. (68), *Nematodirus* sp. (67) and *H. contortus* (69). Because of the low oxygen tensions usually prevailing in the intestinal tract, the high affinity for oxygen of the hemoglobins of these nematodes may be of considerable physiological significance. At very low oxygen tensions oxyhemoglobin may supply oxygen to the tissues of the worms while at higher oxygen tensions reoxygenation of hemoglobin occurs readily. The ability of intact *Ascaris* to reduce the oxyhemoglobin of its body wall under anaerobic conditions has been demonstrated by Davenport (67). The same author has shown that under conditions of oxygen deficiency intact *N. muris* (68) reduces its oxyhemoglobin. When oxygen is readmitted rapid reoxygenation of hemoglobin occurs. Similar observations have been made by Rogers (70) with *N. muris*, *Nematodirus* sp. and *H. contortus*. However, not all the hemoglobins of nematodes can supply oxygen to the tissues of the worms; under anaerobic conditions the oxyhemoglobin of *Strongylus* sp. and that of the perienteric fluid of *Ascaris* are not reduced *in vivo* (67, 68). The occurrence of a hemoglobin in *F. hepatica* has been reported (71), but no information is available about the properties of this pigment.

In a previous review (1) reference has been made to reports concerned with the presence of proteolytic enzymes in a variety of helminths. More recently Cavier (30) has found proteolytic activity in the perienteric fluid of *Ascaris*. As yet, no investigation has been carried out on the purification, the characterization, and the kinetics of proteolytic enzymes of helminths. It is noteworthy that appreciable concentrations of free amino acids have been detected in several helminths (66).

In a recent investigation Rogers (72) has found that peptides, urea, and ammonia are the main nitrogenous products excreted by *Nematodirus* sp. and by *A. galli*. By the use of homogenates of these worms he obtained evidence which suggested that in these parasites urea is formed through the citrulline cycle. Homogenates of young *A. galli* remove purines, possibly by the actions of uricase, allantoinase, allantoicase, and urease.

Biological oxidations.—An analysis of the available experimental data has led to the conclusion that among helminths there are considerable variations

in their dependence on respiratory metabolism (1). With some helminths the requirement for oxidative reactions is high, and short periods of anaerobiosis or partial inhibition of their oxygen uptake results in their death. Others can survive when the oxygen tension of their habitat is extremely low, or when their respiration is inhibited almost completely. These differences are illustrated by the variations in the survival time of helminths under anaerobic conditions *in vitro* [see (2), Table 36, pp. 182–83]. In helminths a rather close correlation appears to exist between the effect of respiration on their rate of fermentation and their requirement for oxidative metabolism. The higher this requirement the more pronounced is the reduction in the rates of fermentation and of carbohydrate utilization produced by oxidative metabolism (1). Recent observations by Ward (73) have shown that under aerobic conditions the rate of carbohydrate utilization in the acanthocephala *M. hirudinaceus* is only slightly lower than anaerobically, indicating a low requirement of this helminth for respiratory metabolism. Measurements of the oxygen tensions prevailing in the intestinal tract close to the mucosa (74) and of the oxygen uptake at these tensions by a number of small intestinal nematodes (75) indicate that respiratory metabolism of these helminths in their habitat may proceed at a fairly high rate. It has not been ascertained whether in these helminths the rates of fermentation are affected by respiration. Evidence for the oxidation of pyruvate through the tricarboxylic acid cycle in these organisms has been obtained by Massey & Rogers (76). The findings of the same authors (77) suggest that fluoroacetate inhibits the oxidative metabolism of these helminths by a mechanism similar to that occurring in mammalian tissues, i.e., by a condensation with oxaloacetate resulting in the formation of fluorocitrate (78, 79). Previous observations had suggested that in the tissue helminth *L. carinii*, fluoroacetate inhibits pyruvate oxidation via the tricarboxylic cycle (80).

Further evidence has been obtained that the requirement of adult *S. mansoni* for oxidative metabolism is low despite the fairly high oxygen tension of the parasite's habitat. The adult worms survive in the host and continue to produce eggs under conditions which result in a marked reduction of their respiration (19, 81). On the other hand, the worms appear to be less resistant to inhibition of glycolysis, an anaerobic metabolic process (82). At an earlier stage of development, i.e., two weeks before maturation, dependence of schistosomes on aerobic metabolism is greater (81). While the adults of *S. mansoni* are considerably more resistant to anaerobiosis and to inhibition of their oxygen uptake than many other helminths, this difference may be a quantitative rather than a qualitative one; it is probable that the parasite has a small, but ultimately essential requirement for respiratory metabolism. Although it can survive *in vitro* in the complete absence of oxygen for five days, survival under aerobic conditions has been observed for considerably longer periods of time (83). It is of interest that, in contrast to the mature worm, cercariae of *S. mansoni* are extremely sensitive to lack of oxygen and die within 1 hr. under completely anaerobic conditions, while at low

oxygen tensions (approximately 6 mm. Hg) their survival is not affected (84). Therefore, it is evident that the requirement for oxidative metabolism varies at different stages of the life cycle of *S. mansoni*.

Evidence for the presence of the cytochrome system in helminths has depended upon spectroscopic observations and on the use of metabolic inhibitors. It has been pointed out (1) that observations made with these procedures do not necessarily prove the occurrence of cytochrome-*c* or of cytochrome oxidase in these organisms. Assays for the catalytic activities of these enzymes supply more conclusive evidence for their presence or absence. Such assays have been used recently in an attempt to obtain more precise information about the mechanisms of biological oxidation in helminths (85). No cytochrome-*c* activity could be detected in homogenates of muscle or of the female reproductive system of *A. lumbricoides*. The possibility was ruled out that the absence of cytochrome-*c* activity was caused by the binding of this respiratory pigment to insoluble tissue particles. Even if the concentration of cytochrome-*c* in the muscle were ten times lower than that of mammalian skeletal muscle it would have been detected by the method of assay. A procedure of even higher sensitivity was used for the measurement of cytochrome oxidase activity which was not detectable in the tissues of *Ascaris*, supporting earlier conclusions of Herrick & Thede (86). Similarly, no cytochrome-*c* or cytochrome oxidase activities could be found in the filarial worm, *L. carinii* (85). Homogenates of the trematode, *S. mansoni*, contain measureable cytochrome-*c* and cytochrome oxidase activities, but they account for only a small fraction of the overall oxygen consumption of the worms. Therefore, respiration in the above three helminths is mediated predominantly, if not exclusively, by respiratory catalysts which are not identical with the cytochrome-*c*-cytochrome oxidase system. On the other hand, Read (87) has demonstrated high cytochrome oxidase activity in the cestode, *H. diminuta*; also, his observations indicate the presence in this worm of a succinoxidase system similar to that of mammalian tissues. In addition, experiments of Massay & Rogers (76) suggest that cytochrome-*c* is, at least in part, involved in the oxidative metabolism of the nematodes *A. galli* and *Nematodirus* sp. It is evident that no generalization can be made about the presence or absence of the cytochrome system in helminths and that this problem must be investigated separately in each individual species. It should be noted that oxidative phosphorylations can occur in *Ascaris* muscle in spite of the absence of the cytochrome system from this tissue (88). Furthermore, *Ascaris* muscle contains a succinoxidase system which differs from that of vertebrate tissues, because transfer of electrons to atmospheric oxygen occurs without the participation of the cytochrome system and because hydrogen peroxide rather than water is a reaction product (85). Possibly a flavin enzyme is a constituent of this enzyme. The substrate for this succinoxidase is supplied by the perienteric fluid of *Ascaris* which contains a heat stable dialyzable factor (or factors) which stimulate the oxygen uptake of washed *Ascaris* muscle (89, 85). It has been found that this effect is, at least in part,

due to the presence of succinate which has been identified as a constituent of the perienteric fluid (10).

Nutrition

As in the case of bacteria, information about the nutritional requirements of helminths can be obtained from the effects of addition and of withdrawal of chemically defined substances (e.g., amino acids, vitamins, etc.) upon the survival and the development of these organisms *in vitro*. Since very few experiments of this type have been carried out, virtually nothing is known about this subject. On the other hand, many helminths can be cultured for varying periods of time outside the host in media containing tissue fluids, extracts of animal tissues, of yeast, and of bacteria. With some helminths, development from one stage of the life cycle to the next has occurred *in vitro*, and in the case of one nematode, *Neoaplectana glaseri*, the entire life cycle of the parasite can be reproduced in culture media (90, 91, 92). The observations which have been summarized and reviewed by Hoeppli *et al.* (93), by Hobson (94), Smyth (95), and Von Brand (2, pp. 204–11) supply a basis for future investigations designed to replace gradually biological materials with chemically defined compounds and to obtain information about the nutritional requirements of helminths which may differ quantitatively and qualitatively from those of the host. This would afford opportunities for a rational development of anthelmintic agents which inhibit the utilization of nutrients essential to the parasite without injury to the host.

A prerequisite for the study of the nutrition of helminths is the elimination of contaminating bacteria which may form toxic substances or which may produce or compete for nutritional factors essential to the worms. In the case of tissue helminths whose habitat is free of bacteria it merely is necessary to use aseptic techniques for the removal of the worms from their surroundings and for their transfer into sterile culture media. On the other hand, intestinal helminths live in surroundings rich in bacteria. In addition, the alimentary canal of intestinal nematodes harbors a bacterial flora. Several methods for the elimination of bacteria from the surface of *Ascaris* have been reported (4, 49, 96, 97, 98). However, even when the medium surrounding the intact worm shows no evidence of bacterial growth, either on visual inspection or on subsequent subculture on aerobic and anaerobic nutrient agar, frequently the intestinal tracts of *Ascaris* are contaminated (4). Following incubation of ascarids in media containing high concentrations of various antibiotics, the intestinal tracts of a relatively large percentage of worms become free of bacteria (4, 49). It has not been determined whether similar results can be obtained with other bactericidal procedures (96, 97, 98).

From observations on the occurrence of water soluble vitamins in helminths it may be inferred that, as in other forms of life, these substances are precursors for coenzymes involved in various metabolic reactions of these parasites. However, such findings do not supply any indications as to whether

the worms can synthesize these vitamins or whether and to what extent they constitute an essential nutritional requirement. Chance & Dirnhuber (99) have found that thiamine, niacin, pantothenic acid, and riboflavin occur in *F. hepatica Moniezia benedeni*, *A. lumbricoides*, and *N. muris* in concentrations which are of the same order of magnitude as those present in the livers of their hosts. The concentration of pyridoxine is higher in the worms, and these authors suggest that, because of the role of pyridoxal in amino acid metabolism, their finding may have a relationship to the high rate of egg production of these worms. The observations of Chance and Dirnhuber regarding the occurrence of riboflavin in the tissues of *Ascaris* have been confirmed recently (100). A number of reports deal with the presence of vitamin C in helminths (101 to 104), but some of the methods used have been subjected to criticism (105, 106). Participation of vitamin-containing enzymes in metabolic reactions of helminths has been demonstrated; for example, that of diphosphothiamine in the production of acetylmethylcarbinol by *L. carinii* (22), that of pyridoxal phosphate in transamination reactions occurring in *F. hepatica* (65), and that of diphosphopyridine nucleotide in glycolysis by *S. mansoni* (10).

A requirement for potassium, calcium, and magnesium ions by the larvae of *Ascaris* has been demonstrated by Fenwick (107). Davey (108) has found that the intestinal nematode, *Ostertagia circumcincta*, has a critical requirement for potassium and calcium, but magnesium ions have no effect on the survival of this helminth *in vitro*.

According to observations of Hopkins, the glycogen content of the plercocercoids of *Schistocephalus solidus* decreases markedly during maturation in the gut of the final host (109). This author has made an attempt to investigate the conditions under which maturation associated with a similar reduction in glycogen occurs *in vitro* (110).

Cavier & Savel (98) have reported that adult *A. lumbricoides* survive for two to three weeks in a buffered salt solution which contains glucose as the only organic constituent. A significant reduction in the time of survival occurs when glucose or when potassium, calcium, and magnesium are omitted. It has not been determined which of these ions are required. Such long periods of survival in simple buffered salt solutions indicate a remarkable ability of *Ascaris* either to store or to synthesize essential nutrients. On the other hand, the trematodes, *F. hepatica* and *S. mansoni*, survive briefly under these conditions. Stephenson (111) has maintained *F. hepatica* in a glucose containing, buffered salt solution for only 40 hr. Omission of glucose reduces the survival time by 50 per cent. Glucose can be replaced by maltose or galactose, while lactose and sucrose are ineffective. Slightly longer periods of survival are observed when fructose is used instead of glucose. *S. mansoni* usually survives for similarly short periods of time in a synthetic medium consisting of salts, glucose, a number of amino acids, purines, and vitamins (83). When certain liver fractions containing protogen are added, survival of the worms is increased up to five days. After these observations had been

made, pure protogens (thioctic acid, α- and β-lipoic acids) have been prepared (112, 113, 114, 115, 116, 117, 118). These compounds are completely ineffective in supporting the survival of *S. mansoni*. Therefore, the nutritional factor essential for schistosomes is not identical with protogen but is present in less purified fractions of this material. Survival of the schistosomes *in vitro* may be increased to a total of three to five days when proline and norleucine are added to a synthetic medium (119). These trematodes have a high requirement for calcium and magnesium. Purines and pyrimidines added as free bases or as their nucleosides have no effect, but, frequently, survival of the schistosomes is prolonged in the presence of ribonucleic or desoxyribonucleic acid (119). Since this effect is not observed consistently, the amount of nucleotides stored by the worms may vary considerably. The critical requirement of the flukes for magnesium may be due to the essential role of this ion in the glycolysis of *S. mansoni*.

<center>CHEMOTHERAPY</center>

This section deals with the chemotherapy of infections caused by helminths which inhabit or invade the gastrointestinal tract, tissues, or blood of man, and represents the major contributions made in this field since World War II.

Intestinal helminths: Cestodes.—Until 1940, the agent most widely used for the elimination of tapeworms from the gastrointestinal tract of man was oleoresin of aspidium. However, consistently good results were not obtained because of great variance in dosage, methods of administration, and general nonavailability of fresh, potent preparations. The optimum schedule for this drug involves a low-residue, semi-starvation diet for 24 to 48 hr. prior to treatment, the administration of a saline purge the night before, and the introduction via duodenal tube of an emulsion containing 6 to 10 gm. oleoresin of aspidium, 8 gm. powdered acacia, 30 gm. sodium sulfate and 100 cc. water. The principle disadvantage of this schedule is the difficulty of successful duodenal intubation, the necessity of hospitalization, and the potential toxicity of the drug, which may produce severe poisoning (headache, vertigo, nausea, vomiting and diarrhea, hyperactive reflexes, jaundice, protein-uria, convulsions, visual disturbances, and respiratory and cardiac failure).

In 1940, Culbertson (120) reported the elimination of the tapeworm *H. fraterna* from mice by the administration of atabrine (quinacrine hydrochloride). This drug had widespread application as an antimalarial during World War II, and soon reports of its efficacy in human taeniasis appeared. Nino (121) reported 34 of 50 subjects having *Taenia saginata* cured with a single treatment of acranil (an acridine dye closely related to atabrine), and Berberian (122) noted equally good results with acranil in *H. nana* infections in children, apparently curing 15 out of 25 children with a large initial dose followed by smaller doses for three additional days.

Using atabrine itself, relatively good results were reported from its oral

administration in infections with *T. saginata*, *T. solium*, *H. nana*, and *Diphyllobothrium latum* by Neghme (123), Shookhoff (124), and Halawani *et al.* (125). Failures were attributed to improper preparation of patients, and two instances of vomiting were noted. Successful results in *T. saginata* infections were reported with single or multiple small doses given during 1 hr. totalling 0.8 gm. of atabrine by Saccomanno (126) (4 out of 4 cures) and Rivero Nogueira (127) (14 out of 14 cures). Hernandez Morales (128) treated 24 patients with 0.6 to 0.8 gm. atabrine and recovered complete *T. saginata* worms from 13. Ten of the remaining subjects were also considered cured because of consistently negative follow-up observations, while one patient passed segments two months later. Nausea and vomiting occurred frequently, and one patient was hospitalized because of severe vomiting and psychosis, from which complete recovery was noted within 24 hr. More recently, Hoekenga (129), Schapiro (130), and Sodeman & Jung (131) reported their experiences with 35, 34 and 11 cases of *T. saginata* infection, respectively, treated with 0.6 to 1.2 gm. atabrine. Complete worms with head attached were recovered immediately after treatment from 24, 21, and 10, respectively, of the above patients treated. The only failure reported by Sodeman & Jung (131) was cured by retreatment, whereas the remaining patients, who did not pass the scolex, treated by Hoekenga (129) and Schapiro (130), were presumed to have been cured because of uniformly negative follow-up observations for 5 to 10 months.

The principal experience with atabrine in the therapy of tapeworms deals with *T. saginata*. Although very few reports are available relative to its use against *T. solium* and *D. latum*, references have been cited (124, 125) wherein successful elimination of these parasites have been reported following atabrine therapy. In the experience of one of the authors (H.M.) *D. latum* has been found readily susceptible to atabrine therapy. Although successful results with atabrine in the treatment of infections with *T. solium* have been reported (125), a wider experience than is at present available will be required before one can evaluate atabrine versus *T. solium*.

With regard to *H. nana*, atabrine has not had extensive trial or study, and the results are not uniformly encouraging. Hoekenga (129) noted only temporary disappearance of eggs in five children treated, and Schapiro (130) reported elimination of adult *H. nana* worms from only 2 of 16 children treated with atabrine. Beaver & Sodeman (132) treated eight subjects with *H. nana* infections, and, although only 37.5 per cent were cured, a high percentage of the worms were removed from those whose stools continued to be positive.

Hexylresorcinol has been reported to be very effective in eliminating *T. saginata* if introduced into the duodenum as an emulsion containing 1.0 gm. crystalline hexylresorcinol, acacia, and water. No prior preparation is required, and no purgative is given afterward. Hernandez Morales & Santiago-Stevenson (133) treated 28 patients in this manner and reported cures in 78.6 per cent on the basis of their failure to observe segments in the stool

during a one year follow-up period. The remainder passed segments and were re-treated, resulting in a secondary cure rate of 80 per cent. The principal advantage of this method over aspidium is that no preparations in diet or purgative are required and that hexylresorcinol is readily available and less toxic than aspidium. However, intraduodenal intubation is required. Atabrine has been used intraduodenally and has been found more effective than hexylresorcinol. Hornbostel & Dorken (134) cured all of 20 male patients treated with 0.8 gm. atabrine dissolved in 100 cc. of water, administered through a duodenal tube. Results in women patients were not as good, because in many severe vomiting interfered with the treatment. In the case of *T. solium* infections, it would appear to be distinctly undesirable to produce gradual disintegration of segments by hexylresorcinol because of the potential danger of developing cysticercosis. In this infection, failure to recover the scolex requires careful follow-up observation and re-treatment until the whole worm has been passed.

Thymol and *p*-cymene have been studied extensively in Finland against *D. latum* infections. Vartrainen (135) treated 68 patients with 1 to 6 gm. thymol in sugar-coated pills, but observed cures in less than 50 per cent. The drug had no effect on *T. saginata* or *A. lumbricoides* infections and produced nausea and epigastric burning with the larger doses. *p*-Cymene administered to 100 *D. latum* patients cured only 28 per cent despite doses as high as 10.0 gm.

Tin preparations were used in the eradication of tapeworms in the days of Paracelsus, and its successful use in Germany, Italy and England was intermittently described later by a number of writers. It fell into disrepute because of toxic reactions presumably caused by the presence of arsenic and lead. Recently, Hirte (136) has reported the successful use of an agent containing tin, tin oxide, and chloride, in 58 cases of *T. saginata* infection. The drug was administered in tablets over a period of five days. Two recurrences were observed after three months and no undesirable or toxic effects on the patients were noted. Purgation with magnesium sulfate was prescribed the day before and after completion of treatment. This simple and apparently effective schedule of treatment has not yet been corroborated elsewhere. If the observations reported are confirmed, it would be very valuable in at least *T. saginata* and *D. latum* infections.

It is apparent from the foregoing reports that atabrine is a highly active anthelmintic and that its administration will result in the immediate and definitive elimination of *T. saginata* and *D. latum* from at least 70 per cent of treated patients harboring these worms. This percentage is based on the recovery of intact worms and scolices immediately after therapy and is probably very much higher if one considers the large number of treated patients who fail to pass segments months after treatment. The principal advantage of atabrine over aspidium resides in the fact that it is a readily available, chemically stable substance which can be given orally without resort to hospitalization or duodenal intubation and that, presumably, it is

much less toxic than aspidium. The major drawback encountered with the use of atabrine as a taeniafuge is the severe vomiting which may follow the administration of 0.6 to 1.0 gm. during the period of 1 hr. The occurrence of severe vomiting may reduce the usefulness of atabrine in the treatment of *T. solium* infections, since it is possible that the anterograde regurgitation of eggs and segments may result in generalized systemic cysticercosis. It has been suggested that vomiting may be avoided or minimized by the administration of sodium bicarbonate and sweet liquids with each dose. Apparently, the best therapeutic results depend on suitable preparations prior to treatment and vigorous purgation afterwards.

Intestinal helminths: Nematodes.—(a) *Ascaris lumbricoides:* Until hexylresorcinol was introduced as an ascaricide in 1930 by Lamson, Ward & Brown (137), the drug of choice in the treatment of ascariasis was oil of chenopodium. The latter drug, however, although still used extensively in many economically underdeveloped areas is now considered potentially too toxic in the face of the proved activity and availability of hexylresorcinol. During the past 20 years, this drug has had widespread trial and acceptance and is now considered the drug of choice in the treatment of ascaris infections. A single dose of 1.0 gm. administered in the fasting state, and followed by a saline purge in 4 hr., will result in removal of approximately 80 per cent of the worms, based on egg counts before and after treatment, and definitive cures in 30 to 90 per cent of patients treated. In addition, hexylresorcinol simultaneously produces significant reduction of egg count and some definitive elimination in hookworm infections. Compared to chenopodium, hexylresorcinol has two drawbacks: one, its relative costliness, and, two, the fact that it must be given in a special "crystoid" capsule to prevent the caustic action of the drug on the mucous membranes of the mouth. Since a great proportion of ascaris infections occur in children and infants, many of whom cannot swallow a capsule, numerous attempts have been made to develop an agent which can be given as a liquid or tablet.

Ascaridole, the active principle and toxic constituent of oil of chenopodium, has been synthesized and tested clinically in ascaris infections (138, 139). Its mode of action and cure rates resemble those of oil of chenopodium, and the principal virtue of this agent is the fact that it can be prescribed in exact amounts. Oil of chenopodium contains 40 to 80 per cent of ascaridol, and, presumably, erratic therapeutic results and toxicity may be related to uncertainty of dosage. However, the potential toxicity of ascaridol despite possibly superior results with maximal doses of ascaridol compared to oil of chenopodium is a serious drawback and offers no significant advantage over hexylresorcinol.

Hetrazan (1-diethylcarbamyl-4-methylpiperagine hydrochloride), a piperazine drug widely used in filiariasis, has recently been introduced as an ascaricidal agent, and numerous reports have appeared attesting its value in the treatment of ascariasis. The initial reports were in conflict concerning the usefulness of hetrazan, but, in the light of more recent clinical evalua-

tion, it is evident that early failures were related to inadequate dosage and duration of treatment. Hewitt & Mazzotti (140), for example, found that of 47 school boys given a single dose of 3 to 13 mg./kg., 15 passed no worms, and, although the remainder passed one to seven worms, their stools remained positive. Colbourne (141) likewise reported failure with doses of 2 mg./kg. three times a day for one day. On the other hand, Koekenga (142) who observed cure rates of about 35 per cent from treatment with hetrazan given in a single dose or divided doses for one day, reported 60 per cent cures after the treatment was extended to three days (200 mg., three times daily). Later (143), he reported cure rates of 80 per cent following 12 mg./kg. per day for four days, and Ettledorf & Crawford (144) also noted good results (73 per cent cures) following 6 mg./kg. three times daily for one week. The most comprehensive recent study has been reported by Loughlin *et al.* (145). Hetrazan was administered in syrup form in a single dose for three and four days, and quantitative egg counts were carried out before and seven and fourteen days after completion of treatment. The authors believe a single daily dose of 13 mg./kg. for four days to be an optimum schedule for syrup of hetrazan. Of 37 patients who received the latter treatment, 20, or 54 per cent, were cured, but, in the remaining 17 patients, the egg counts were reduced by 56 to 99 per cent. No effect was noted on other coexisting helminths. Only one patient of a total of 104 who received at least 13 mg./kg. of hetrazan for three or four days exhibited any evidence of toxicity. This consisted of anorexia, vertigo, and depression, and was considered insignificant and as not requiring cessation of therapy. It is probable that extending therapy to five or even seven days would result in markedly enhanced therapeutic efficacy.

It is apparent from the foregoing that hetrazan has marked anthelmintic properties. Its principal value is in the treatment of infants and children under circumstances which permit the administration of drugs for at least four days. Under these circumstances, hetrazan as a syrup can be given to children and infants without fear of toxicity, without dietary restrictions or purgation, and with assurance that at least 50 per cent will be cured and the density of the infection in the remainder significantly reduced. Under field conditions, and for mass treatment, it may not be as practical as a single dose of hexylresorcinol whenever gelatin capsules or specially prepared tablets can be given. Nevertheless, hetrazan represents a distinct and valuable addition to agents available for the treatment of ascariasis.

(*b*) Hookworms: The drugs most widely used in the treatment of hookworm infections are oil of chenopodium, carbon tetrachloride, hexylresorcinol, and tetrachlorethylene. Oil of chenopodium and carbon tetrachloride, frequently administered together, are undoubtedly more toxic than either hexylresorcinol or tetrachlorethylene and are not recommended, although still used extensively in the field and for mass treatment because they are cheap and can be given in liquid form, whereas tetrachlorethylene and hexylresorcinol are given in gelatin capsules. It is generally conceded by

clinicians that the drug of choice at the present time for the treatment of hookworm infections is tetrachlorethylene. However, in the tropics, where hookworm and ascaris frequently occur in the same patient, it is a common practice to treat both simultaneously with hexylresorcinol, although the latter is not as effective against the hookworm as tetrachlorethylene. Nevertheless, several courses can be given at weekly intervals, as a result of which definitive cure of ascaris may result, and the density of the hookworm reduced below clinical significance. The literature dealing with the use of hexylresorcinol and tetrachlorethylene is too voluminous for detailed review, but it may be stated conservatively that both agents are useful in hookworm infections and that reduction in egg output varies from 60 to 100 per cent with either drug, depending on the initial density of the infection, tetrachlorethylene being more effective (75 per cent) in *Necator* infections.

During World War II, it was noted that many troops with hookworm infections acquired in various Pacific areas still were passing eggs despite as many as eight or nine courses of tetrachlorethylene. Most *et al.* (146) reported a study in which treatment of hookworm with tetrachlorethylene was evaluated on the basis of the species of worm recovered. One hundred sixty nine patients not treated previously were given tetrachlorethylene. Sixty nine were found to harbor *Necator*, 87 *Ancylostoma*, and in 13 both were present. A single course of treatment was found to cure only 25 per cent of *Ancylostoma* infections, whereas 66 per cent of the *Necator* infections were cured from a single dose of 3.0 cc. It is evident, therefore, that this drug is not as effective against *Ancylostoma* as it is in *Necator* infections, and an evaluation of this agent must take into account the species of parasite as well as the density of infection. Cashew (*Anacardium occindentale*) nutshell oil, which experimentally has been found to remove 90 to 100 per cent of *Ancylostoma* from dogs, has been studied in humans by Eichbaum *et al.* (147). Its effectiveness is lower than that of hexylresorcinol or of tetrachorethylene.

(*c*) *Strongyloides stercoralis:* Unfortunately, no satisfactory drug is available which can be counted on to eliminate this infection from a significant number of patients. The drug most widely used is gentian violet, and, although larvae may disappear for variable periods of time, definitive cures are accomplished rarely. The drug as currently available is a tablet so coated as to delay disintegration for several hours and, actually, until it is beyond the site of localization of the worms A standard course consists of the administration of 0.06 gm. three times daily for 16 days, but it has been suggested that the efficiency of gentian violet may be enhanced if the dose is gradually increased until the standard is trebled. Frequently, severe vomiting and diarrhea occur even with the lower dosage schedule, and in the experience of one of the authors (H.M.), the therapeutic effects from either regimen are negligible. The administration of gentian violet intraduodenally or intravenously has been recommended, but there are no reasonably well-documented clinical series of cases from which one can determine the value, if any, of this procedure.

(*d*) *Trichocephalus trichiurus:* Whipworm infections, unless very severe, are often considered nonpathogenic clinically and are not usually treated because effective drugs are not available. The agent reported to be most effective since 1929 is the latex of one or more species of Central and South American *Ficus* trees, and locally known as *leche de higueron.* The active principle of the latex is ficin, a proteolytic enzyme which cannot be used in the pure state clinically because of the possibility of digesting through a mucous membrane which is not entirely intact or normal. Thus, the use of the fresh crude latex is limited to those areas where the proper trees grow. Nevertheless, recent studies with *leche de higueron* by Hoekenga & Box (148) in Panama indicated egg reductions of only 60 per cent, whereas previous studies by Caldwell & Caldwell (149) and Faust (150) indicated greater efficiency. It may be that the discrepancies reported are related to variance in age and potency of different samples of *leche de higueron.*

Emetine hydrochloride administered by mouth in specially coated tablets has been found very effective in eliminating whipworms. Burrows *et al.* (151) reported elimination of 88 per cent of the worms from 23 whipworm infected patients in a mental hospital. Shrapnel (152) also reported good results from "enseals" of emetine hydrochloride. However, this drug in the enteric coating used has not been made available commercially because of the toxic experiences noted during treatment. Nausea, vomiting, and diarrhea were observed, and, not infrequently, blood and bits of mucous membrane were detected in the stools. However, the bleeding is not serious, representing slight hemorrhage related to the dislodgement of the anterior portion of the worm from the mucous membrane in which it is attached. Therefore, the use of emetine is of value in eliminating whipworms where there is a good clinical indication to justify treatment. The upper intestinal toxicity from emetine can be avoided and its therapeutic efficiency enhanced if the drug is given in solution rectally in a dose of 1 mg./kg. after a prior cleansing enema (153).

More recently, a hexylresorcinol emulsion containing gum acacia and barium sulfate has been reported by Basnuevo *et al.* (154) to be effective in the treatment of whipworm infections. The drug must be introduced through the rectum by a rubber catheter which is inserted for at least 15 cm. and the filling of the colon verified by fluoroscopic examination. This procedure appears to be impractical for routine adoption, although Jung & Beaver (155) reported excellent results from simple water retention enemas of 0.1 to 0.2 per cent hexylresorcinol (500 to 700 cc.) after prior cleansing irrigation. Faust (150) believes the most effective safe treatment in the United States is the oral administration of 2.7 cc. tetrachlorethylene and 0.3 cc. oil of chenopodium after prior purgation and colonic irrigation. It appears that the latter treatment in conjunction with emetine or hexylresorcinol enemas is most effective in reducing the density of trichiurus infections below clinical significance.

In 1937 the administration of ferric ammonium citrate was reported to result in the cure of a high proportion of trichiurus infections (156, 157). There has been no confirmation of these observations. In a limited number

of hospitalized patients the administration of 3 to 5 gm. of ferric ammonium citrate for 14 days did not result in any definitive cures (158) on the basis of daily egg counts.

(e) *Trichinella spiralis:* No drugs are known which influence the biology of *Trichinella* infections in man. Huge doses of hetrazan (600 mg./kg. per day for 5 to 10 days) were reported by Oliver-Gonzalez & Hewitt (159) to reduce the density of both adults and larvae in experimentally infected rats. Magath & Thompson (160), on the other hand, could not confirm these observations with doses of 50 and 100 mg./kg. administered intraperitoneally for 14 to 21 days. In either case, the doses used are 10 to 40 times those used in man for the treatment of filariasis or ascariasis. The variations in the clinical course of trichinosis are such that any beneficial results noted in a few human cases treated with hetrazan could not be unequivocally related to the drug. While one of us (H.M.) has observed defervescence of fever in some trichinosis patients receiving this drug, he also observed no effect even with doses of 1.0 to 1.5 gm. daily.

Recently, Davis & Most (161) have shown that ACTH may modify the clinical response of a patient critically ill with severe trichinosis to permit ultimate recovery. Histologic studies of muscle obtained before, during, and after therapy, as well as quantitative determinations of the degree of larval involvement indicated that ACTH did not influence the nature or severity of the inflammatory reaction nor the parasitic or serologic evolution of the infection. Presumably, the acute allergic and toxic phases of the disease were controlled, and a striking reduction of fever and clinical improvement occurred. Undoubtedly in this case, ACTH was a life saving measure, and the use of this agent in severe trichinosis infections is recommended.

(f) *Enterobius vermicularis:* Although many drugs have been introduced periodically for the treatment of pin worm infections, the status of the therapy of this infection is still unsatisfactory. This largely results from the persisting nature of this widespread infection as well as from the fact that the drugs currently in use or available are still ineffective or too toxic.

Gentian violet was introduced by Wright *et al.* (162) in 1938 as an effective drug in the treatment of pin worm infections, and in 1940 (163) a more extensive report was published, indicating cure rates in the range of 85 per cent. Since then, gentian violet has been used extensively, and is generally considered the drug of choice in the treatment of pin worm infections. The principal disadvantages attending the use of gentian violet lie in the frequent occurrence of minor though distressing symptomatic toxicity in the form of abdominal cramps, nausea, vomiting, and diarrhea, in the fact that effective treatment requires administration of the drug for 16 days during a period of 23 days, and, finally, in that the subjects most frequently treated are young children, many of whom cannot take the prescribed gentian violet tablets.

Phenothiazine has been used in the treatment of *Enterobius* infections since 1940, and numerous investigators have confirmed Manson-Bahr's (164)

original successful results. At least 1500 patients with *Enterobius* infections have been treated by Most (165), Sisk (166), Bercovitz *et al.* (167), Miller & Allen (168), and Kiutunen-Ekbaum (169). Although there have been marked variation in total dosage and duration of therapy, all investigators reported moderate to excellent results with cure rates ranging from 75 to 95 per cent. However, despite the efficacy of phenothiazine, its use in the treatment of such a relatively nonpathogenic infection as enterobiasis is not recommended because of the occurrence of hemolytic anemia, drug hepatitis, and one reported fatality.

Various carbamate derivatives have been used for about 15 years as therapeutic agents in enterobiasis. Berberian & Dennis (170) treated 60 enterobiasis patients with 0.3 to 0.9 gm. Lubisan (m, n-butoxy-phenyl diethyl carbamate) daily for three days, but in spite of early parasite clearance in a high proportion of cases, 77 per cent were found positive again within five weeks after treatment. Sisk (171) reported 72 per cent of 51 patients cured of pin worms after two three-day courses of Lubisan with daily doses as high as 1.2 gm. No evidence of toxicity was noted. Berberian in yet another study (172) reported cure rates of from 17 to 44 per cent following therapy with benzyl-phenyl carbamate (butalan, diphenan) of 0.45 to 1.5 gm. daily for three to ten days. Similarly poor results have been reported following treatment with Egressin (thymyl *n*-isoamylcarbamate) (173, 174). Goddard & Brown (174), for example, reported cure rates of only 40 per cent from a two-day course of egressin given to 28 children and 15 adults. Minor toxic symptoms (abdominal distress and light-headedness after tobacco and alcohol) were noted in several patients. There are, on the other hand, numerous papers, principally in the European literature, attesting the value of these carbamate anthelminthics. Nevertheless, the evidence deduced from carefully followed patients indicates that none of the carbamate compounds studied to date are sufficiently active to justify their use in preference to more active compounds, unless the latter, gentian violet for example, cannot be tolerated. Under such circumstances, several four-day courses of Lubisan (1.0 gm. daily) or one or more two-day courses of egressin are recommended with no great probability of cure.

The most significant recent advance in the chemotherapy of enterobiasis is the demonstration by Wells (175) of the effectiveness of terramycin in eliminating pin worms from patients. Sixty-one infected individuals in 16 families were treated with terramycin, which was used for 18 days. The standard therapy was not followed by all individuals, but there is little doubt that the drug was very effective because all 12 infected members of two families who took the prescribed amount of terramycin remained negative during an observation period of $2\frac{1}{2}$ months after treatment. In the remaining patients, only two failures occurred within four weeks of treatment, whereas the later failures were considered reinfections. Approximately 30 per cent of the patients experienced marked diarrhea during treatment, but in spite of this the patients considered terramycin more tolerable than gentian

violet. In a subsequent communication, Loughlin *et al.* (173) reported the successful elimination of the infection for at least five weeks from 52 patients treated with terramycin for five days (10 gm. total adult course). It is well known that pin worm infections usually involve several members of a family or many individuals in institutionalized populations, and unless all are treated simultaneously universal cure will not be accomplished. The numerous parasitic relapses (approximately 40 per cent) which have now been reported following terramycin probably represent reinfections, since these have occurred after the fifth week, which is the outer limit for the life cycle of this parasite. The optimum dosage and duration of treatment has not been determined, but Young (176) has found that 2.0 gm. of terramycin daily for four days resulted in definitive cures in at least 20 institutionalized children followed for 18 weeks after treatment. Shookhoff (177), in a limited number of cases, has noted that patients treated with duomycin (aureomycin) likewise lose their pin worm infections for at least five weeks.

The status of pin worm therapy can be summarized briefly by stating that gentian violet is very effective but produces too much minor toxicity and frequently cannot be given to young children and infants, that phenothiazine is potentially too toxic, that several carbamate compounds, notably Egressin and Lubisan, while only slightly effective, are easily tolerated, and that terramycin is at present the drug of choice.

Filiariasis and Schistosomiasis.—A comprehensive bibliographical review of these infections prepared by Brown (178) and Brady (179) respectively in 1949 was published in 1951. The reader is referred to their papers for a summary and evaluation of chemotherapeutic agents used in the treatment of the various filarial and schistosomal infections. The current review presents a concise evaluation of the status of the therapy of these infections at this time.

(a) Filariasis: Many investigators have demonstrated that a host of different chemical substances are effective in varying degree, notably in *Wuchereria bancrofti* infections, in reducing the number or causing the disappearance of microfilariae from the peripheral circulation. With the exception of Suramin sodium (Bayer 205) and hetrazan, the drugs found effective, namely ethylstibamine (neostibosan), stibamine glucoside (neostam), urea stibamine, stibanose, stibophen (fuadin), anthiomaline, tartar emetic, monosodium antimony thioglycollate, arsenamide, melarsen oxide and mepharsen are derivatives of antimony or arsenic. Of the antimony compounds, ethylstibamine is probably least toxic and most effective resulting in definitive elimination of microfilariae from 60 to 75 per cent of patients treated and reduction of 90 to 99 per cent of microfilariae in the remainder. Arsenamide and melarsen oxide are probably the least toxic and most effective of the arsenicals tested, and their use has been reported to result in elimination of larvae from 75 per cent of the patients treated and reduction in number of microfilariae by 70 to 99 per cent in the remainder. The maximum effect following the use of the heavy metals is not manifested until months after

completion of treatment, and the slow disappearance of microfilariae probably represents their natural longevity and reflects the sterilization of the adult worms. However, the development of tender nodules in the groin or scrotum or lymphangitis or both during or after treatment, as well as the demonstration of inflammatory changes in surgically excised nodules, suggests major deleterious effects or death of adult worms in some cases. Nevertheless, the toxicity attending the use of heavy metals, especially arsenicals, as well as the disadvantages of numerous intramuscular or intravenous injections, almost excludes these agents as suitable for mass therapy in filariasis.

Hetrazan (1-diethylcarbamyl-4-methylpiperazine) was discovered and introduced as a therapeutic agent against filariasis in 1947. This drug can be given by mouth, is relatively nontoxic except in *Onchocerca* infections, and has an extremely rapid action in removing microfilariae from the blood. The pharmacology and experimental and clinical studies with hetrazan in the various filarial infections of man and other mammals have recently been reviewed by Hawking (180). From his own data, as well as from that of others reviewed by him, and from more recent publications, one can conclude as follows: (*a*) Hetrazan is extremely effective in destroying microfilariae which occur in the blood, e.g., *W. bancrofti*, *Loa loa*, and *Dirofilaria immitis*. It is less effective against larvae of *Onchocerca* in skin and ineffective against microfilariae of *W. bancrofti* in hydrocoel fluid and of *W. volvulus* in nodules. Hawking believes this failure is due to absence of macrophage cells which in other locations seize and dissolve the microfilariae after they have first been changed in some way by an opsonin-like action. (*b*) Adult worms in general are poorly or not at all affected by hetrazan. It is agreed by many that hetrazan causes the death of some *Loa* adults but probably most adult *Onchocerca* are not affected by treatment. In the case of *W. bancrofti*, the occurrence of nodules, fever, eosinophilia, and lymphangitis during or shortly after treatment in some patients suggests death of some adult worms.

It is obvious from the foregoing that the clinical application of filaricidal drugs, including hetrazan is extremely limited. In the case of *W. bancrofti*, it is questionable that any clinical benefit will result if the principal effect of hetrazan is on the microfilariae, which in themselves produce no symptoms or signs of disease. Although symptomatic and objective improvement has been reported in bancroftian elephantiasis following the use of hetrazan, it is difficult to visualize alteration in irreversible anatomic changes associated with elephantiasis unless an occasional adult worm is dislodged from an obstructed lymphatic space. On the other hand, one can likewise visualize obstruction induced by death of worms caused by hetrazan, followed by severe inflammatory reaction in the involved lymph node, space, or vessel. In the case of onchocerciasis, the infection obviously cannot be eradicated from the host because of the failure of hetrazan to affect the adult worms. The destruction of the larvae in the skin and eye may temporarily result in control of cutaneous and ocular symptoms. Unfortunately, however, severe

allergic reactions which may be systemic or limited to the eye, skin, or sub-
cutaneous tissues occur frequently following the initial administration of
hetrazan to patients with *Onchocerca* infections. In *Loa* infections, it is
generally agreed that hetrazan causes death of some worms and that by
repeated courses of treatment the infection may be controlled clinically.

The most attractive feature of hetrazan therapy appears to be in its mass
application in villages or entire islands to prevent new infections by reducing
the numbers of microfilariae available to the arthropod vectors. Consider-
able success in this direction against *W. bancrofti* has been reported from
Africa, the Caribbean, and the Pacific. In the case of *Onchocerca*, however,
control with hetrazan alone cannot be accomplished because larvae reappear
in the skin soon after hetrazan therapy. These observations have been re-
ported by Burch (181) who also found that Suramin (Bayer 205) was more
effective against adults of *Onchocerca* than hetrazan, and that a combination
of both drugs was more effective than either one in producing death of both
adults and microfilariae. The combined treatment is limited to individual
clinical cases for obviously repeated courses of hetrazan alone or combined
oral and parenteral administration of Suramin are not practical control
measures. It appears that, in spite of the extreme effectiveness of hetrazan
against microfilariae, this drug does not offer the practitioner an agent which
he can readily use to terminate the infection, to regularly or consistently
control the symptoms or signs of filariasis, or to prevent the sequelae of
adult worm infections. In conjunction with arthropod control measures, and
possibly along with other drugs, the principal value of hetrazan appears to
be in its application in the field of public health.

(*b*) Schistosomiasis: Despite the intensive clinical and experimental
chemotherapeutic studies in the treatment of schistosomiasis during the past
35 years, relatively little progress has been made in the control of this in-
fection. In 1918, Christopherson (182) demonstrated the usefulness of tartar
emetic against schistosomiasis, and, in 1929, Khalil *et al.* (183) introduced
stibophen (fuadin), a sulfonated catechol-antimony complex; since then the
principal and most widely used drugs in the treatment of schistosomiasis
have been these two antimony compounds. The major contributions and
advances have been related to the establishment of the relative value of
tartar emetic and stibophen against *S. japonicum*, *S. mansoni*, and *S.
hematobium*, the optimum schedule of administration and total dosage of
these compounds, the parenteral and oral trial of other trivalent as well as
pentavalent antimony compounds, and the discovery in Germany during
World War II of Xanthone and Thioxanthone derivatives highly active in
experimental primate schistosomiasis. A comprehensive review of the litera-
ture, as well as the personal experience of one of the authors (H.M.) permits
the following brief evaluation of the current status of the therapy of schisto-
somiasis.

At the present time tartar emetic is the most effective drug in the treat-
ment of the three human schistosomal infections. Total doses of 2.5 gm.,

1.8 gm., and 1.5 to 1.75 gm. will cure more than 90 per cent of infections due to *S. japonicum*, *S. mansoni*, and *S. hematobium*, respectively. The principal disadvantages attending the use of tartar emetic are that the drug requires care and experience in administration, is moderately toxic, and must be administered intravenously during a relatively long course requiring numerous injections. Despite these objections, tartar emetic is the drug of choice in the treatment of infections by *S. japonicum*. The pharmacology, toxicity, schedules of administration, and comparative efficiency of tartar emetic and stibophen in this infection have been reported by Most *et al.* (184) in studies conducted during World War II. They found that a standard course of 40 cc. of stibophen (6.3 per cent solution) cured only about 10 to 15 per cent of patients with *S. japonicum* infections. If the dose was increased to 70 cc., cure rates were only about 25 to 30 per cent, whereas an equivalent amount of antimony contained in 1.45 gm. tartar emetic cured approximately 50 per cent. If the total dose of stibophen on a schedule of 5 cc. every other day is increased to 100 cc. the cures reach only 40 per cent, whereas if the same amount of antimony is given as tartar emetic at least 90 per cent of cures are observed. It is obvious as a result of these observations that tartar emetic is superior to stibophen in the treatment of schistosomiasis caused by *S. japonicum*. It is also apparent that *S. japonicum* is less sensitive to antimony than are either *S. mansoni* or *S. hematobium* because doses of 70 to 100 cc. of stibophen which cure only 30 or 40 per cent of *S. japonicum* result in cures of at least 75 per cent of the other infections. Further, if 5 cc. of stibophen are given every day to a total of 100 cc., almost 100 per cent of *S. mansoni* infections may be cured.

With regard to so-called intensive treatment, i.e., schedules in which total doses are markedly increased or the period of therapy contracted to a few days, there is evidence that the cure rates in *S. mansoni* and *S. hematobium* infections are significantly enhanced. A two-day course of 6 to 8 injections of sodium antimony tartrate (12 mg./kg. total dose) was introduced by Alves (185) in 1945 in Southern Rhodesia. Since then, several hundred patients have been treated and followed for several months, cure rates of 75 per cent or more being observed. In the experience of one of us (H.M.) this schedule, as well as a continuation of this daily dosage for five days, was totally ineffective against *S. japonicum*. Further, trials in Egypt were found so toxic that this method had to be discontinued. Similarly, although 8 cc. of stibophen daily for 13 days is superior to the standard course of 100 cc. extending over three to four weeks, the method was too toxic for general use. In short, the so-called intensive schedules, while improving the therapeutic results, are too toxic and impractical for mass control measures.

Other trivalent and pentavalent antimony compounds (ethylstibamine, stibamine glucoside, urea stibamine, anthiomaline and stibanose) have been found moderately effective in *S. mansoni* infections and ineffective in *S. japonicum*. On the whole, on the basis of toxicity in some instances and of the need for intravenous medications in others, it is questionable whether

any offer advantages over stibophen. Antimony compounds, notably anti-
mony trigluconate and butyl antimony gallate, while moderately effective
in animal trials orally, have been almost totally ineffective and too toxic
for human use by mouth.

During World War II, Kikuth, Gönnert & Mauss (186) developed a series
of xanthone and thioxanthone derivatives which were effective orally in
experimental schistosomiasis. These compounds, principally miracil, miracil
D, their naphthoate, salicylate, and other derivatives have had limited or ex-
tensive trials in various parts of the world. A most interesting report present-
ing original work as well as a review of the status of these agents has been
made recently by Newsome (187). Apparently most investigators who have
studied these drugs have agreed to their clinical effectiveness principally in
S. hematobium, and less so in *S. mansoni*, infections. However, there have
been major differences in therapeutic results and toxic experiences, and, in
the light of recent studies, these appear to be related to the preparations or
derivatives of miracil studied, the total dose and schedules of administration,
differences in severity of infection and exposure to re-infection in various
geographic areas, and, most important, differences in criteria of cure. New-
some, on the basis of his observations, which included hatching and rectal
biopsy techniques in evaluating cure after treatment, concluded that
"miracil D has a marked curative effect on *S. hematobium* while the effect on
S. mansoni is perhaps less pronounced." Calculated actual cure rates appear
to be 39 per cent and 27 per cent respectively for these infections, although
clinically most patients appear to be cured. The results in Africa and South
America appear to be better than in Egypt, but in this country the results
would probably be better if the drug could be given as two three-day courses
in the order of 100 to 125 mg./kg. Coated tablets have been found less toxic
than the uncoated hydrochloride of miracil and undesirable side effects are
also reduced if the naphthoate or salicylate derivatives are used.

From the foregoing, it appears that tartar emetic administered intra-
venously in total doses of 2.5 gm. according to the schedule of Most *et al.*
(184) is the drug of choice in the treatment of *S. japonica* infections. This
schedule should cure at least 95 per cent of patients. If, because of toxic
manifestations or the necessarily prolonged period of hospitalization, this
schedule is not feasible, then daily intramuscular injections of 5 to 8 cc. of
stibophen for at least 20 consecutive days are recommended. The latter
course of treatment will cure approximately 60 to 75 per cent of the infec-
tions, depending upon the size of the daily dose tolerated and the total dose
given. In the treatment of individual cases of *S. hematobium* and *S. mansoni*
infections, 5 cc. of stibophen intramuscularly daily are recommended with
total doses of 75 per cent and 100 per cent respectively for the above infec-
tions from the standpoint of definitive cures approaching 100 per cent. For
alleviation of symptoms or mass control schedules for large population
groups, the miracil derivatives are recommended with the reservation that
the actual cures will be low. The so-called intensive two- to three-day anti-

mony schedules and the oral antimonials presently available are either too toxic or insufficiently active to favor their adoption except for experimental purposes. Unfortunately, a nontoxic oral preparation which is curative for all forms of human schistosomiasis is not available.

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FOOD POISONING[1]

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The term food poisoning as used in this review applies in general to gastrointestinal disturbances following the ingestion of food containing certain bacteria or their products. The exception to this definition is botulism which may or may not be accompanied by gastrointestinal disturbances. Since the mode of action of the living agents in causing symptoms is not understood, the term food poisoning will suffice until more information becomes available.

The causative agents of bacterial food poisoning fall into two categories. In the first of these the organisms themselves are harmless when introduced into the gastrointestinal tract. When these microorganisms contaminate a food in which they can grow they elaborate a toxin under suitable time and temperature relationships. The toxin and not the organism is responsible for illness when such food is eaten. Examples of this type of food poisoning are botulism and staphylococcus food poisoning. In the second category certain living organisms in sufficient numbers may cause illness. Causative agents of this type of food poisoning are *Salmonella*, *Streptococcus faecalis*, certain strains of *Escherichia coli*, *Bacillus cereus*, and possibly others.

CLOSTRIDIUM BOTULINUM

Clostridium botulinum is a spore forming anaerobic saprophytic microorganism. The interest in *C. botulinum* stems from the disease which follows the eating of food containing the toxin of the organism. Little is known about its metabolism as it exists in the soil in competition with other microorganisms. Since the spores of *C. botulinum* are in the soil, and hence in dust, all foods are subject to contamination with these spores. If, in food preservation, food is sufficiently heated to destroy many of the naturally occurring microorganisms but not all of the spores of *C. botulinum*, the surviving spores may germinate and produce toxin. Germination of spores in such food is dependent on many factors, such as pH, moisture (3), and the concentrations of salt, sugar (4), and rancid fatty acids (5).

Susceptibility of man and animals to the toxins.—Five types of *C. botulinum* have been distinguished on the basis of the toxins which they produce. These are A, B, C, D, and E. The toxin of each type is different and gives rise to a specific antitoxin when injected into suitable experimental animals. The biochemical properties of some of the types are also different (2).

The symptoms produced in experimental animals receiving oral or paren-

[1] The survey of the literature pertaining to this review was concluded in December, 1952. The review is limited to recent developments in the field, and the readers are referred to textbooks by Dewberry (1) and by Dack (2) for broader coverage of the subject.

teral injections of the toxin of the five types are the same for all. Different animal species vary in their susceptibilities to the toxins of the various types. Burgen, Dickens & Zatman (6) determined the LD_{50} of Type A and Type B toxins by intraperitoneal injection of 200 gm. white rats. They found the rat LD_{50} for Type A toxin to be equal to 25 mouse LD_{50} and for Type B toxin equal to 10,000 mouse LD_{50}. Moll & Brandly (7) fed mink concentrated toxin of *C. botulinum* types A and B and found that 20 to 80 million mouse M. L.D.'s (intraperitoneally) were required to produce symptoms of botulism. Quortrup & Holt (8) demonstrated that mink were highly susceptible to the toxin of Type C *C. botulinum*. Dinter & Kull (9) described three outbreaks of type C botulism in mink on farms in Sweden. Kull & Moberg (10) point out the difficulty in the laboratory diagnosis of botulism in mink. The concentrations of toxin in the tissues may be too small to make a diagnosis possible and oftentimes the stomachs of necropsied animals may be empty. Yet they definitely proved that the cause of death in 12 of 22 minks which came from different mink farms in Sweden was Type C botulism. These workers also demonstrated the value of immunizing mink with Type C botulinum toxoid.

Macaca mulatta are susceptible when fed toxins of Types A, B, and E (11). The spores of Type C *C. botulinum* are more resistant to heat than are those of Type E, and for that reason outbreaks of botulism due to Type C might be expected. However, in man outbreaks of botulism caused only by Types A, B, and, more rarely, E have occurred. The reason why no human outbreaks of Type C botulism have been reported is at present not apparent.

Botulism.—Prevot (12) isolated a strain of *C. botulinum* which he classified as Type D from horses during an epidemic of what was at first thought to be meningoencephalitis. The toxin of this strain has been studied by Boroff, Raymond, & Prevot (13) who found that it was not neutralized by horse antitoxin against Types A, B, and C but was neutralized by antitoxin prepared against the Belgian strain of *C. botulinum* Type D. The toxin of this strain has been obtained in colloidal (insoluble at pH 7) and soluble forms. The soluble toxin was reported to be split off the colloidal particle by the action of ultrasonic waves in the presence of catalase. No outbreaks of botulism resulting from the toxin of Type D have been reported for man. Much more study is needed of Type D *C. botulinum* to establish its relative toxicity in different animal species.

Dolman and co-workers (14) have described an outbreak of Type E botulism in man, attributed to home-pickled herring. Of particular interest is the fact that fresh herrings inoculated intramuscularly with the Type E strain of *C. botulinum* supported growth and toxin production within 72 hr. when stored at 23 C. Furthermore, these authors summarized five outbreaks of botulism in North America caused by Type E. They pointed out that the prototype E strains were originally isolated from fish in the U.S.S.R. Four out of five outbreaks of Type E botulism were from preserved fish either canned, smoked, or pickled.

Toxin of Type A Clostridium botulinum.—Of the five types of botulinum

toxin only Type A toxin has been crystallized. Type B toxin has been highly purified, leaving an amorphous end product. The crystalline Type A toxin was shown by Lamanna (15) to possess hemagglutinating activity, and subsequently Lamanna & Lowenthal (16) showed that the hemagglutinin and the toxin were separate entities. Further studies on the effect of heat, formalin, and acid on the toxicity and hemagglutinating activity of *C. botulinum* Type A culture supernates by Lowenthal & Lamanna (17) confirmed the previous findings of the nonidentity of the two properties.

Lamanna & Lowenthal (16) demonstrated two components in crystalline Type A botulinum toxin by means of the Oudin test and showed that the hemagglutinin could be removed by agglutinin-absorption technics leaving only one component, the toxin.

Mode of action of C. botulinum toxin.—Masland & Gammon (18) studied the effect of botulinum toxin on the electromyogram of the guinea pig and the cat. Local botulism was produced by intramuscular injection of toxin; this was evidenced by muscle flaccidity without fibrillation. Prostigmine failed to reverse the action of toxin, as is the case in curare poisoning.

Torda & Wolfe (18a) claimed that choline acetylase is strongly inhibited by the toxin and that this might explain how the toxin produces neuromuscular block. In contrast Burgen *et al.* (6) found that botulinum toxin exerted no inhibitory effect on the acetylization of choline by either fresh or acetone dried rat brain. They studied botulinum toxin in the rat phrenic nerve-diaphragm preparation. While they were unable to localize with certainty the site of action of the toxin, their evidence could be explained by an interaction of toxin with the fine unmyelinated nerve fibers entering the end-plate region, thus causing a block in the transmission of the nerve impulse. However, in the iris, the adrenergic fibres are non-myelinated throughout their length; whereas the cholinergic fibres are myelinated, except probably at their endings; and also it has been shown that botulinum toxin acts preferentially upon the cholinergic fibres that are myelinated up to their endings (19, 20, 21). Ambache (19) injected botulinum toxin into isolated intestinal segments of mice and observed a loss of peristaltic activity and of motor response to nicotine. The inhibition of responsiveness to small doses of nicotine was abolished by large doses of ephedrine, which abolishes the inhibitory action of adrenaline. Two distinct types of ganglion cells in the myenteric plexus were postulated; stimulation of one, consisting of cholinergic fibers, caused contraction of the intestine. Ambache (20) in a later study examined the susceptibility to botulinum toxin of a number of different nerves of the autonomic system of the cat. He produced paralysis of the ciliary ganglion by retrobulbar injections of toxin and located the lesion in the preganglionic fibers. In still other experiments Ambache (21) studied the effect of toxin injected into the superior cervical ganglion on one side in 16 cats. The integrity of the ganglionic transmission was examined 20 to 48 hr. later in the following two pathways: (a) that to the dilator papillae; (b) that to the nictitating membrane. With adequate doses of toxin a complete

preganglionic paralysis occurred in the papillo-dilator pathway, but a proportion of nictitating membrane synapses are able to withstand the paralytic action of the toxin, even in large amounts.

Spores of C. botulinum.—Many factors influence the formation of spores of *C. botulinum*, and no methods have been devised for producing uniformly high yields of spores in laboratory media. Wynne (22) measured the effect of three salts, Na_2SO_4, NaCl, and KCl on the sporulation of Type A *C. botulinum* and found an exponential relationship between concentration of the salts and absolute numbers of spores produced. Blair (23) found that the omission of methionine from a synthetic medium suppressed spore formation of *C. botulinum*.

Effect of antibiotics on spore germination.—Penicillin has been shown by Wynne (22) to have no effect on the germination of spores of *C. botulinum*, and similarly, Collier & Wynne (24) found no effect on germination of the spores of *C. botulinum* with streptomycin. In the case of subtilin Andersen (25) found that 0.4 p.p.m. prevented as many as 517 spores of *C. botulinum* Type A from producing colonies in pork infusion agar. One p.p.m. subtilin permitted 253 out of 800,000 spores to produce colonies. Cameron and Bohrer (26) inoculated canned peas with spores of *C. botulinum* Types A and B. The canned peas containing 20 p.p.m. subtilin were inoculated with 1000 and one-half million spores per can. The cans were heated to 93°C. Botulinum spoilage occurred at both levels of inoculum in the case of Type A and at only the higher inoculum level in the case of Type B.

Williams & Fleming (27) found a difference among strains of *C. botulinum* in sensitivity to subtilin in culture medium although there was no difference in behavior of Types A and B. They found the time required for appearance of growth from spores to be increased with an increase in subtilin content combined with large inocula. Their studies indicated that subtilin was sporostatic rather than sporocidal.

Effect of fatty acids on germination of spores.—Foster & Wynne (28) found that oleic, linoleic, and linolenic acids inhibited germination of spores of *C. botulinum*. Roth & Halvorson (5) however, found that these fatty acids and their methylesters were inhibitory only when rancid. Benzoyl peroxide inhibited in the same order of magnitude as rancid, unsaturated fatty acids. Catalase only partially reversed the inhibition produced by the rancid fatty acids.

Factors affecting heat resistance of spores.—Sugiyama (29, 30) found that spores of *C. botulinum* formed at 37°C. are of higher heat resistance than those developed at 41, 29, or 24°C. The addition of Ca^{++} and Mg^{++} to the spore suspending menstruum was found to reduce the heat tolerance of the spores. The NaCl concentration of the sporulating medium had no significant effect on the heat resistance of the spores. Below a certain concentration, the lower the Fe^{++} and Ca^{++} concentration of the sporulating medium, the lower the thermostability of the spores. The thermal resistance of spores suspended in different concentrations of sucrose was found to increase with

the concentration of sucrose. Spores suspended in 50 per cent sucrose show an increase almost immediately in heat resistance and this remains high for many hours. Fatty acids in the sporulating medium influence the heat toler-ance of spores. In general the longer the fatty acid chain, the greater is the increase in thermal tolerance. Sugiyama found it necessary to remove the excess fatty acid salts from the spore suspension in order not to suppress germination.

STAPHYLOCOCCUS FOOD POISONING

Feig (31) in studies of outbreaks of food poisoning reported to the United States Public Health Service for the years 1945 to 1947 observed that 82.2 per cent of those in which the etiological agent was determined were due to staphylococci. Occasionally repeated outbreaks have occurred from food processed in a single establishment. Food poisoning staphylococci have been shown to persist for months in the noses of certain individuals (2). Duguid & Wallace (32) studied two subjects who carried coagulase positive staphylo-cocci in their anterior nares. They found that the air was infected more regularly and to a greater degree by the raising of dust from clothing than by sneezing. Their studies, if applied to carriers of enterotoxin producing strains of staphylococci, indicate the ease with which staphylococci may contaminate food other than by handling and by contaminated utensils and cutting boards.

Hauge (33) made a thorough study of an outbreak of staphylococcus food poisoning which he traced to mashed potatoes containing unpasteurized milk kept warm for 8 hr. before serving. The milk came from two cows, one of which was shedding 2500 staphylococci per ml. of milk. Hauge gave a comprehensive review of the significance of staphylococci as a cause of mastitis in dairy cattle and pointed out the staphylococcus food poisoning hazard in dairy products.

Evans & Niven (34) were unable to detect a difference between the physiologic activities of the enterotoxigenic staphylococci and those coagu-lase-positive cultures that failed to cause vomiting when fed to monkeys. Evans, Buettner, & Niven (35) fed cultures of 24 coagulase-negative staphy-lococci isolated from foods suspected of causing food poisoning to *Macaca mulatta* monkeys. In no instance was enterotoxin found. Enterotoxin pro-duction was detected in four of five coagulase-positive strains isolated from wholesome frozen foods and in five out of seven coagulase-positive strains isolated from disease processes in man.

Frog test for assay of staphylococcus enterotoxin.—Robinton (36, 37) de-scribed a rapid test for the demonstration of staphylococcus enterotoxin consisting of intragastric injection into decerebrated frogs (*Rana pipiens*). According to Robinton antiperistalsis of the stomach occurs only when known emetic or enterotoxigenic substances have been fed. Such reactions were reported to follow in from 1 to 30 min. after intragastric injection de-pending upon the concentration of the enterotoxin. Eddy (38) was unable to

confirm Robinton's work and concluded that the occurrence of a spasm in frogs fed staphylococcal culture products is either a nonspecific response to various substances of an especially viscid nature, elicited more readily at certain seasons, or is a response at a particular season to some undetermined stimulus. Surgalla, Bergdoll, & Dack (39) fed to 19 intact and 85 decere-brated *Rana pipiens* enterotoxin or various nontoxic control materials. The reactions of frogs to enterotoxin were inconsistent and unrelated to dosage, and positive reactions occurred in frogs fed saline and other nonenterotoxic materials.

Staphylococcus enterotoxin.—Large volumes of staphylococcus entero-toxin have been successfully produced in hydrolyzed casein mediums (40). It has been produced in shallow cultures in Roux bottles, in deep cultures in turning bottles, and in deep aerated cultures. The enterotoxin has been concentrated and partially purified (40a) by concentration in vacuo, dialysis, and precipitation with ammonium sulfate, ethanol, and methanol. Enterotoxin was found to withstand hydrochloric acid at pH 3.5 for a period of 22 hr. at 4°C. Ethanol in a 65 per cent concentration appeared to weaken the toxin when stored for 24 hr. at 4°C. There was no diminution in toxin stored in saturated ammonium sulfate for 24 hr. at 4°C. The partially puri-fied toxin did not withstand boiling for 30 min. at pH 6.0 or 7.5. The proper-ties of enterotoxin suggest that it is protein in nature.

The concentrations of antigens in enterotoxic solutions have been tested using a modification of the technic of Oudin (41). A simple technic has been developed for direct identification of antigens responsible for bands appear-ing in different tubes. This test is of value in following the chemical purifica-tion of enterotoxin although at the present time no definite band has been proven to be caused by enterotoxin.

Costlow, Cone, & Garey (42) produced enterotoxin by growing food poisoning staphylococci in a medium containing glucose, iron-free acid, hydrolyzed casein, thiamin, niacin, biotin, tap water and phenol red indica-tor. The cultures were incubated in an environment of from 25 to 45 per cent carbon dioxide for three days at 37°C. The pH of filtrates from these cultures ranged from 5.5 to 5.9. No toxicity of these filtrates was demon-strated by either intravenous injections or feeding of kittens unless the pH was adjusted to 7.0 to 7.5. No toxic effects were demonstrated with uninocu-lated medium treated in the same manner.

SALMONELLA FOOD POISONING

Gastrointestinal disturbances due to *Salmonella* are caused by the living organisms and not by preformed toxic products of the organisms. The habitat of these bacteria is the intestinal tract of man and animals. In an extensive investigation Mackel *et al.* (43) found that 244 (15 per cent) of 1626 normal dogs studied carried *Salmonella* representing 41 types. Among domestic animals used as food for man *Salmonella* types are more common in

swine and poultry than in cattle or sheep. A survey of this problem has been made by Hinshaw & McNeil (44).

Although food poisoning outbreaks caused by *Salmonella* are uncommon compared to staphylococcus food poisoning, nevertheless they are of importance from the public health standpoint. Many of the recent studies of *Salmonella* have been concerned with egg products, such as liquid whole eggs and spray-dried eggs. In these products *Salmonella* types without clearly established pathogenicity for man have been described.

McCullough & Eisele (45, 46, 47) have fed human volunteers strains of *Salmonella* isolated by Solowey and co-workers, (48) from market samples of spray-dried whole egg powder. Their studies were made with three strains of *S. meleagridis*, three of *S. anatum*, one each of *S. newport*, *S. derby*, and *S. bareilly*, and four of *S. pullorum*. When a sufficient number of the organisms were fed all strains caused illnesses in human volunteers. The requisite dosage varied with the strain within the type. In the case of one strain of *S. anatum*, illnesses were caused when 587,000 organisms were fed, whereas with another strain of the same type 44,500,000 organisms were required. In the case of *S. pullorum* a billion or more organisms were required to cause illness, but the authors call attention to the fact that the volunteers fed these cultures received periodic booster injections of typhoid vaccine which contains the same somatic antigen as *S. pullorum*. Further work is required to settle the problem of whether or not immunization of man with killed suspensions of typhoid bacilli may confer protection against infection with *S. pullorum*.

Incubation period.—In the studies of McCullough & Eisele (45, 46, 47) the incubation period from the ingestion of the cultures to the onset of symptoms ranged from $3\frac{1}{2}$ hr. to 7 days. In a series of 433 individual feedings there were 73 illnesses; in three cases (4.1 per cent) the incubation period was less than 5 hr., while in 42 (57 per cent) of the 73 cases, the incubation period was 24 hr. or less.

Immunity following infection with Salmonella.—McCullough & Eisele (49) carried out refeeding experiments on 23 subjects who had become ill following the ingestion of either *S. meleagridis* or *S. anatum*. Increased resistance was found in all 23 although the degree of insusceptibility varied. Of seven subjects who developed prolonged carrier states in the initial experiments, six promptly ceased excreting organisms in the refeeding experiments.

Pathogenicity of Salmonella strains for man and mice.—McCullough (50) studied three strains each of *S. meleagridis* and *S. anatum* and one strain each of *S. newport*, *S. bareilly*, and *S. derby*. The pathogenicity of these strains was established for human volunteers and the LD_{50} was determined for mice using the intra-abdominal route. There was no relationship between pathogenicity of these strains for human volunteers and the LD_{50} for mice.

Salmonella in egg products.—Cantor & McFarlane (51) and Chase &

Wright (52) have rarely found liquid whole egg produced from chicken eggs under laboratory conditions to be contaminated with *Salmonella* other than *S. pullorum*. Solowey, Spaulding, & Goresline (53) consider the external egg shell surface as the important source of contamination with *Salmonella* which are introduced unknowingly into the liquid whole egg by egg-breaking personnel.

Pasteurization of liquid egg products to destroy Salmonella.—Schneider (54) showed that the commercial practice of preheating liquid whole egg (140°F. and above) prior to spray-drying effectively reduces the incidence of *Salmonella* organisms in whole egg powder. Goresline *et al.* (55) attempted to eliminate *Salmonella* from egg products by pasteurization. Milk pasteurization equipment was found satisfactory for pasteurization of liquid whole egg, and it was found that with careful attention to operating procedures the liquid whole egg could be flash-heated to 140°F. and held at that temperature for 3 min. to kill *Salmonella* without appreciably affecting the qualities of the egg product. Winter (56) recommends pasteurizing of whole egg and yolk at 144°F. for 1 min. or 142°F. for 2 min.

Anellis, Lubas, & Rayman (57) determined the heat resistance of some strains of *Salmonella* in liquid eggs. The organisms were suspended in liquid egg and sealed in thermal death time glass tubes for heating. The antigenic type and strain and the pH of the liquid egg in which the organisms were suspended were all factors affecting thermal resistance of the organisms. At least twice as much time at constant temperature was required for bacterial destruction at pH 5.5 than at pH 8.0. Osborne, Straka, & Lineweaver (58) studied the heat resistance of several strains of *Salmonella* in liquid whole egg, egg white and egg yolk. They observed a shorter thermal death time in the pH region 8 to 9 than in the region pH 5.5 to 7.5. All of the strains which they tested were more stable in liquid whole egg than in phosphate buffer or egg white. One strain of *S. senftenberg* was found to have a severalfold longer thermal death time than any other strain of *Salmonella* tested.

ENTEROCOCCI

Alpha-type streptococci in large numbers have been found in foods incriminated in outbreaks of food poisoning where no other causative agents could be detected (2). In all instances where the biochemical properties of these strains have been studied, the organisms were found to be identical with enterococci. Illnesses have frequently occurred in human volunteers fed strains grown in suitable media. The symptoms are usually mild, consisting of abdominal cramping and diarrhea and, less commonly, nausea and vomiting.

Streptococcus faecalis has been used in producing cheddar cheese by Dahlberg & Kosikowsky (59). This strain, like other strains of the same species, decarboxylates tyrosine to form tyramine. Human volunteers were fed this strain as well as chemically pure tyramine to evaluate possible hazards which might arise from eating cheddar cheese made with the specific strain of *S.*

faecalis (60). Cheese made with this strain as well as cultures of the starter strain were fed to volunteers without effect. Tyramine monohydrochloride, fed to human volunteers in 0.3 and 1 gm. amounts in one pint of milk, caused no effect upon blood pressure nor did it produce any sign of illness. In these experiments illnesses did not occur when old cultures isolated from food poisoning outbreaks were fed. This raises the question whether cultures lose the property of causing illness under prolonged laboratory cultivation or whether the type of food in which they are found in outbreaks is of significance. Certainly more work is needed to clarify the role of *S. faecalis* in food poisoning.

ESCHERICHIA COLI

The gram-negative intestinal bacteria when classified on the basis of biochemical and antigenic properties comprise a large mosaic pattern. The *Salmonellas* first were studied intensively, and later attention was given to the *Escherichia coli* group by Kauffmann & Dupont (61). Apparently the ability of these organisms to cause gastrointestinal illness is not limited to particular groups. Attention has been given to the power of certain antigenic species of *E. coli* to cause infantile diarrhea. Taylor, Powell, & Wright (62) and Giles & Sangster (63) in England associated certain antigenic types of *E. coli* with infantile diarrhea. Neter and co-workers (64) fed a strain of *E. coli* serotype 0111 (D433) to a two-month old infant with multiple congenital defects. One hundred million viable organisms were fed with the formula after first demonstrating that this strain was not present in the intestinal tract of the infant. Within 24 hr. diarrhea and weight loss followed, and the serotype (D433) was found in small numbers in the throat and in large numbers in the feces and nasopharynx. When a different type of *E. coli* which was isolated from the infant prior to the experimental feeding was similarly fed no ill effects followed. Two serotypes of *E. coli* have been associated with infant diarrhea, 055 and 0111. Neter and his associates studied clinically and bacteriologically three infants with diarrheal disease and vomiting. Cultures of the stools before illness failed to reveal serotypes 055 and 0111, but during illness one of these two serotypes was found for each of the three infants. Attention is called to the fact that infants may be carriers of these serotypes without showing any signs of illness.

Stevenson (65) isolated a strain of *E. coli*, which he designated 111,B_4, from the stools of 14 adults with diarrhea, but he came to no conclusion concerning the etiological role of the organism. Ferguson & Jure (66) fed a mixture of three serotype strains of *E. coli* 111,B_4 in milk to adult human volunteers. With a dosage of 9×10^9 all of 12 volunteers were made ill; with a dosage of 6.5×10^9 all of 11 volunteers were made ill; whereas with a dosage of 5.3×10^8, 8 out of 12 volunteers became ill. Some of the subjects developed agglutinins for this strain. No illness or agglutinins developed when a strain of *E. coli* from a normal healthy infant was fed. The illnesses were characterized by an average incubation period of from 10 to 12 hours with

one episode occurring 5 hr. after feeding a dose of 9×10^9 viable organisms. The symptoms consisted of nausea, cramps, and diarrhea, and sometimes vomiting. There was usually also an elevation of temperature.

Bacillus Cereus

A number of outbreaks of food poisoning are described in which spore forming gram-positive aerobic bacilli have been found in the implicated food item. Many of these organisms of the genus *Bacillus* have not been identified and their roles as causative agents of illness in the outbreaks are not clearly established.

Hauge (67), a veterinarian in Oslo, has carefully studied several outbreaks which he thought were caused by *Bacillus cereus*. These illnesses were characterized by an incubation period varying from 8 to 16 hours but usually from 12 to 13 hours. The symptoms included nausea, seldom vomiting, and severe cramp-like abdominal pains located in the lower part of the abdomen and around the umbilicus. Tenesmus and frequent cramp-like contractions of the rectum without defecation sometimes occurred. However, watery diarrhea was common in many cases with the passage of from 4 to 6 stools. The illness usually lasted from 6 to 12 hr. and was not accompanied by fever or weakness.

Of four large outbreaks involving 600 persons studied by Hauge, three occurred in hospitals and one in a home for aged people. In one of these outbreaks in which vanilla sauce prepared from a commercial powder seemed to be involved, some of the powder was used experimentally to prepare a vanilla sauce to simulate the conditions at the time of the outbreak. The sauce was stored for one day at room temperature under conditions identical to those preceding the outbreak, and a plate count after 24 hr. revealed 36 million *B. cereus* per ml. By direct microscopic examination 100 to 110 million *B. cereus* per ml. were found. Hauge examined the pathogenicity of the isolated strain of *B. cereus* by inoculating 10,000 organisms per ml. into vanilla sauce prepared with dry-sterilized vanilla sauce powder of another brand. After one day of incubation at room temperature the sauce showed a plate count of 43 million and a direct microscopic count of 92 million *B. cereus* per ml. Hauge ate 200 ml. of the sauce and 13 hr. later developed severe abdominal pain, tenesmus, and frequent watery stools, with no fever or generalized weakness, and with recovery in 8 hr.

Hauge considers potatoes and corn starch to be naturally contaminated with *B. cereus* and warns against leaving foods prepared with these starches at incubation temperatures where they are held for a period before serving. He fed to six human volunteers from 155 to 270 ml. of vanilla sauce containing from 30 to 60 million *B. cereus* per ml. Two of these subjects remained unaffected, two became slightly ill, and two developed pronounced symptoms. Stool examinations in the ill revealed either none or few *B. cereus*.

Christiansen, Kock, & Madelung (68) described an outbreak in a summer

camp in Marseliborg near Arhus where 15 of 18 adults and 106 of 130 children between 8 and 16 years of age became ill following a meal in which yellow pudding was implicated as the cause of the outbreak. Culture of the pudding revealed 13 million *B. cereus* per gm. with no other bacterium growing. Other agents were excluded and the symptoms were typical of those described by Hauge. The yellow pudding had been stored in cups for 24 hr. at a temperature favorable for bacterial growth.

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THE ECOLOGY OF MOSQUITO BORNE VIRUSES[1]

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The ecology of a mosquito borne virus involves a consideration of the growth of the virus within the vertebrate and arthropod hosts, the reaction of these hosts to the presence of the virus, and the vertebrate-arthropod associations which are necessary for the dissemination and maintenance of the virus. Little is known of the physiological properties of a particular host and virus which permit multiplication of the virus. In this review stress will be laid upon studies which attempt to discover the mechanisms in nature which maintain these viruses. The first section will relate to the geographical distribution and time of occurrence of the viruses as indicated by clinical disease in human beings and domestic animals, antibody surveys among various animal species, and attempts at isolation of viruses from arthropods and other animals. Section two deals with transmission and viremia studies with suspected vectors and vertebrate hosts and virus cycles in the laboratory. Section three is concerned with field observations attempting to demonstrate that suspected vectors and their vertebrate hosts occur in sufficient numbers and are so associated that a virus cycle is possible. Section four deals with the problem of maintenance of virus during prolonged cold or dry periods. Section five pertains to serological relationships of interest from the standpoint of virus evolution. Some of the newly described viruses are described in section six.

The review is concerned primarily with the literature of the past five years and in case of yellow fever, with the past two years. Adequate reviews are available for previous literature (1, 2, 3).

Occurrence of Clinical Disease

California virus.—Three possible cases of encephalitis were observed in California in the summer of 1945 by Hammon & Reeves (4).

Western equine and St. Louis viruses.—California:—Lennette & Longshore (5) reviewed 174 confirmed western equine encephalitis cases and 135 St. Louis encephalitis cases occurring in California from 1945 through 1950. These cases were limited essentially to the hot, irrigated agricultural sections of the Central and Imperial valleys. The greatest number of western equine encephalitis cases occurred during July; the greatest number of St. Louis encephalitis cases occurred during September. Although 20 per cent of the western equine encephalitis cases occurred in children under 1 yr., only 1.5 per cent of the St. Louis encephalitis cases were found in this age group.

[1] The survey of the literature pertaining to this review was concluded in March, 1953.

The greatest number of encephalitis cases was in 1950 with 157, the smallest number was 1 in 1948. Although a summer with many western equine encephalitis cases usually showed an increase in the number of St. Louis encephalitis cases, the incidences of the two diseases did not bear any definite relationship to each other. The California State Department of Health (6) reported that from July 5 to November 3, 1952 there were 342 confirmed western equine, and 32 proven St. Louis encephalitis cases. The peak incidence of western equine encephalitis cases was in July, and the peak incidence of St. Louis encephalitis cases was in September.

Colorado:—Twenty-five cases of western encephalitis from northeastern Colorado were reported by Mulder (7) during the summer of 1949.

Missouri:—Smith et al. (8) in 1948 reported that sporadic cases of St. Louis encephalitis had been encountered in St. Louis County every year since the 1937 outbreak.

Saskatchewan:—Laboratory experiences with western encephalitis cases in Saskatchewan from 1939 through 1951 were reviewed by Fulton & Burton (9). In 1939 virus was isolated on two occasions from patients. From 1941 on, neutralization tests were performed on sera from suspected patients. In 1944 there were no positive sera, in the other years the number of positive sera varied from a low of 2 in 1946 to 96 in 1941. There were 25 or more positive sera in 1941, 1942, 1948, and 1949. No serum with St. Louis neutralizing antibodies was found. Gareau (10) reported seeing epidemics of western encephalitis at Regina in 1938, 1939, 1941, and 1947.

Texas:—Four patients with western equine encephalitis were seen by Dravin et al. (11) at the veteran's hospital at Amarillo in the summer of 1949, and also a St. Louis case from New Mexico.

Washington:—A summary of the incidence of acute summer encephalitis in the Yakima Valley from 1938 through 1949 was presented by Reeves et al. (12). In the years 1939 through 1942, the number of reported cases varied from 28 to 51. Since then there has been little reported disease.

In addition to the above reports there were two which present difficulties. A case of western encephalitis was reported from Chicago by Saphir & Milzer (13). This case occurred in May, 1948, and had no known exposure outside of the city. St. Louis virus was isolated by Sanders et al. (14) from the brain of a patient who died in Florida following a chronic illness resembling Guillain-Barres disease. A case of western equine encephalitis as early as May, and a chronic illness attributable to St. Louis virus are both most unusual.

Eastern equine encephalitis virus.—Louisiana:—Milner et al. (15) in August, 1946, isolated the eastern equine virus from a child living in southern Louisiana. Hauser (16) reported ten proven cases occurring during September and October 1947 in southern Louisiana.

Dominican Republic:—An outbreak involving 13 persons in the northern part of the Dominican Republic during late 1948 and early 1949 was reported by Eklund et al. (17).

Venezuelan encephalitis virus.—Ecuador:—Levi-Castillo (18) observed a few cases in 1951 and stated that Venezuelan encephalitis has been a problem in the coastal plains.

Japanese encephalitis virus.—China:—Chao & Chung (19) report that 229 cases of encephalitis were seen during the summer months at two Peking hospitals from 1942 through 1951, 28 prior to 1947 and 201 since. In addition, during the past few years acute encephalitis was reported from Canton and Taiwan in the south, Chungking and Changtu in the west, Shanghai and Nanking in the east, Tientsin, Peking, and Paoling in the north, Sian in the northwest, and Dairen and Mukden in the northeast of China.

Guam:—Hammon (20) reports an outbreak in Guam late in 1947 and early in 1948. This is the first epidemic reported in a tropical region.

Japan:—A small outbreak occurred in the inland sea area of Japan in 1947 (20). In 1948, according to Wyatt (21), a severe epidemic occurred throughout Japan, during which 8000 cases were reported.

Korea:—Epidemics have occurred in Korea in 1934, 1935, 1939, and 1949, according to Hullinghorst *et al.* (22). A few cases were observed in American troops in 1946. From the last week of August to the middle of October, 1949, 5548 cases were reported, and all provinces were affected with the exception of the Island of Cheju-Do. According to Lincoln & Sivertson (23) and Dickerson *et al.* (24), approximately 300 cases occurred among American troops in Korea in the Pusan area from mid-July to mid-October, 1950.

Malaya:—The first case recognized in Malaya occurred near Kuala Lumpur in November, 1951, and was described by Paterson *et al.* (25). Subsequently, a case in a British soldier in Malaya, and three cases in Singapore in children have been reported (26).

Manchuria:—Mitamura *et al.* (27) reported the occurrence of the disease in southern Manchuria in natives in 1945, 1946, and 1947, and in Soviet troops in 1946 and 1947.

Okinawa:—Tigerett & Hammon (28) summarized available data. A history of 68 cases in 1933 was obtained. In each year from 1945 to 1949 inclusive, cases were recognized. The smallest number of reported cases was 32 in 1946, and the largest number was 196 in 1947. Cases occurred from June to September.

Philippines:—In May, June, and July 1946, Salafranca & Espiritu (29) saw 5 patients with central nervous system symptoms and rising antibody titers. Some of the cases appear to have been atypical.

Murray Valley encephalitis virus.—Victoria:—Anderson (30) during the first 15 weeks of 1951, saw 40 cases of acute encephalitis in the Murray Valley district of northern Victoria and the immediately adjacent area of southern New South Wales. It resembled the acute encephalitis which occurred during late summer in western and central New South Wales and in parts of Victoria and Queensland during 1917 and 1918. In 1951 a small number of cases also occurred in South Australia north of Adelaide, and

were studied by Miles *et al.* (31). Derrick (32) has pointed out that acute encephalitis in Australia has not been limited to the Darling Basin. In 1917 the epidemic extended as far as Brisbane and Townsville in Queensland, in 1922 there was an epidemic in South and Central Queensland which did not extend into New South Wales, and in 1925 cases occurred at Townsville as well as New South Wales. Young children at Townsville were affected during 1942. In 1951 cases occurred in several parts of Queensland.

Rift Valley fever virus.—South Africa:—Rift Valley fever was first recognized in the Union of South Africa in the autumn of 1951. The disease was observed in the western portion of the Orange Free State, the Southern Transvaal, and the Northwestern Cape Provinces (33, 34, 35, 36).

Yellow fever virus.—Panama:—Elton (37) reports that sylvan yellow fever appeared east of the Canal near Pecora during November and December, 1948. Cases were then recognized in August and September, 1949, January 1950, and April 1951, the outbreak proceeding toward the Atlantic Coast and the West. In June 1951, the first case was recognized in eastern Costa Rica near the Panamanian border. From late in July until early October there was a severe outbreak in the northeastern part of Costa Rica. In October 1951, yellow fever appeared on the Pacific coast of Costa Rica. In January 1952, a case occurred near the border of western Panama, and in February 1952, a case was recognized just over the border of Panama. In July and August 1952, cases appeared in eastern Nicaragua (38). No case of yellow fever had been recognized in Central America since 1924.

Periodicity in occurrence of yellow fever.—Kumm (39) reviewed the reported occurrence of yellow fever during the first 50 years of this century. Most reported cases were from the Americas. The period of time between successive peaks of high prevalence varied from 2 to 13 years with a median of 6 years. Data from the viscerotomy services of Brazil, Bolivia, and Colombia showed the same periodicity with peaks of high prevalence at 5 to 9 years with a median of $6\frac{1}{2}$ years.

Animal Infections

Eastern equine virus.—United States:—There were two severe localized outbreaks of the eastern type in the past five years among horses; one in Louisiana in 1947, and one in Louisiana and Arkansas in 1949 (40, 41). Over 14,000 horses and mules were reported to have been sick in Louisiana in 1947, and 1525 in 1949, with an undetermined amount of disease among wild horses.

From August 24 to early October 1951, epizootics caused by the eastern virus occurred among pheasants on three breeding farms in Connecticut (42). In New Jersey outbreaks in pheasants were noted once in 1948, and five times in 1951. In Pennsylvania there was one outbreak in 1951 near the New Jersey border. Onset of these epizootics varied from late July to late October (43).

Dominican Republic:—The small outbreak of the eastern type of human

encephalitis in 1948 and 1949 was preceded and accompanied by a severe epizootic among horses and donkeys (17).

Philippines:—Livesay (44) reported the isolation of the eastern equine virus from a sick monkey in Manila during 1947, thus providing the first evidence that eastern virus is present outside of the Western Hemisphere.

Venezuelan encephalitis virus.—Ecuador:—The disease is a problem among horses and mules of the coastal plains of Ecuador (38).

Japanese encephalitis virus.—China:—According to Okubi *et al.* (45), epizootics were observed among horses in north China during the summers of 1940 and 1945.

Japan:—During the summer of 1947, Burns & Matumoto (46) reported 1209 horses affected, the most severe epizootic since 1935 and 1936. Every island was affected except Hokkaido. In southern Japan most cases occurred during July and August, in northern Japan during September and October.

During the summers of 1947 and 1948 a large number of pregnancies in swine termined in still births and sick piglets, from the latter of which virus was isolated. There had been a similar disease among swine during the 1935 epidemic (47, 48, 49).

Malaya:—Paterson *et al.* (25) reported a small outbreak among race horses at Singapore in late July and early August, 1951.

Rift Valley fever virus.—South Africa:—In addition to illness of sheep and cattle, abortion was noted in wild springbuck and blesbuck in the epizootic in the Union of South Africa (33, 34). Much disease in human beings appeared to be a contact infection from autopsies on dead animals.

Human and animal disease is of value as an indicator of virus presence mainly during periods of severe epidemics and epizootics, and even then because of the frequency of subclinical infection it is a poor indicator of the extent of dissemination of virus. During interepidemic and interepizootic periods, antibody surveys and isolation of virus from invertebrate hosts, and occasionally from vertebrate hosts, must be used to detect virus. With most of these viruses, and possibly with all, involvement of man or his domestic animals appears to be a fortuitous event, and search for more fundamental cycles necessary for the existence of the virus must be pursued among other species. Human and animal disease, however, is valuable in demonstrating climatic conditions and geographical areas favorable for the existence of the virus. Also the periodicity in the appearance of such disease must reflect some fundamental variations in the mechanisms supporting the growth of the virus.

ANTIBODY SURVEYS: SERA OF HUMAN BEINGS

California virus.—In Kern County General Hospital from 1944 to 1949, 11.1 per cent of 188 patient sera had neutralizing antibodies. Since there was no evidence that any patient had disease caused by this virus, 11 per cent presumably represents the incidence of antibodies in this county. In 1947 and 1948, the California Health Department Laboratory found 7.8 per cent

of 292 sera with neutralizing antibodies. Of 157 sera from the hot valley areas, 10 per cent had antibodies; of 135 sera from all other parts of the state, 5.2 per cent had antibodies. A small number of sera from Washington, Colorado, North Dakota, Kansas, and Tokyo had no neutralizing antibodies (4).

Eastern equine virus.—Louisiana:—In 1947, 4 of 22 sera from members of families of patients had antibodies, but antibodies were not present in the sera from 23 neighbors (50). In the Dominican Republic in 1949, an incidence of 13 per cent of neutralizing antibodies was found at Monte Christi on the coast. Inland, the incidence of antibodies dropped until 35 kilometers from the coast no antibodies were found (17).

Japanese encephalitis virus.—Japan:—Bowell *et al.* (51) in 1946 obtained sera from people living in four areas of Japan, selected for a north-south representation. Little human disease had been recognized since a severe outbreak in 1939. At Sapparo, in the north, 4 per cent of 233 sera had neutralizing antibodies. At Tokyo and vicinity there were few persons with antibodies under the age of eight. Antibody incidence increased from 51 per cent in the 10 to 14 age group to 92 per cent in the 20 to 39 age group. Above this it was 100 per cent. At Okayama no children under five had antibodies. The incidence increased from 12 per cent in the 5 to 9 age group to 60 per cent in the 15 to 19 age group and reached 98 per cent in the 60-and-over age group. At Kumamoto in the south, the incidence was 11.5 per cent in the age group below 9. It increased to 33 per cent in the 15 to 19 age group, and to 78 per cent at 60 and over.

China:—Mitamura *et al.* (27) reported an incidence of 61 per cent of neutralizing antibodies in 1941 in central China and 44 per cent in French Indo-China.

Korea:—A survey in 1946 by Deuel *et al.* (52) at Seoul and vicinity showed an incidence of 16 per cent in the 1 to 4 age group, a gradual rise to 80 per cent in the 20 to 39 age group, and a slightly greater per cent in older age groups. At Kunson and vicinity the incidence in the 1 to 4 age group was 63 per cent, in the 5 to 9 age group, 85 per cent, and 100 per cent in the age group 20 or over.

Malaya:—In sera collected in Malaya over the period of 1948–50, neutralizing antibodies were found in 23 of 27 native Malayans, and in 9 of 23 others (25).

Manchuria:—Mitamura *et al.* (27) reported a 30 per cent incidence of neutralizing antibodies at Kirin in 1936 and no antibodies at Harbin in 1942.

Okinawa:—Sera obtained in December 1946 from two villages on the west coast where encephalitis had occurred in 1945 showed 27 per cent neutralizing antibodies in the 3 months to 4 year group, 78 per cent in the 10 to 14 age group, and 90 per cent or greater in the age groups over 15 (52).

Philippines:—In 1949 Salafranca and Espiritu (29) reported that 7 of

21 adult Filipinos had sera with neutralizing antibodies. Mitamura *et al.* (27) in 1938 found 1 of 96 people in Manila with antibodies.

Sumatra:—Hammon quotes Kitaoka as having found antibodies in people of Sumatra (20).

Java:—Mitamura *et al.* (27) in 1939 found 2 of 91 people with antibodies.

Murray Valley encephalitis virus.—Victoria:—In 1951 in northwestern Victoria, 20.4 per cent of people over, and 7.5 per cent under 15 years of age had complement-fixing antibodies. In northeastern Victoria approximately 4.5 per cent of people of all ages had such antibodies. In the rest of Victoria, north of the Divide 3.1 per cent and south of the Divide approximately 0.75 per cent, had complement-fixing antibodies. Of sera from Queensland, 24 of 119 had antibodies, from New South Wales 5 of 135, from Western Australia 1 of 55, from New Guinea 13 of 58, from Tasmania 1 of 85, and from New Zealand none of 9 sera had antibodies (53). Of sera obtained early in 1952 none of 85 sera from northwestern Victoria, 1 of 369 from northern Victoria, 3 of 287 from New South Wales, and 2 of 361 from Queensland showed complement-fixing antibodies (54).

Miles & Howes (55) found that 25 of 284 sera from South Australia and the Northern Territory contained neutralizing antibodies, and 12 contained complement-fixing antibodies.

St. Louis encephalitis virus.—Missouri:—Surveys in St. Louis County, Missouri, in 1943 and 1944, showed neutralizing antibodies in the sera of 37 per cent of 97 people living in St. Louis through 1933 and 1937 and in 5.3 per cent of 56 people who had come into the area from the east since 1937 (8).

Rift Valley fever virus.—Smithburn *et al.* (56) in Uganda found neutralizing antibodies in 3 of 101 sera from adults, in none of 65 sera from children.

Antibody Surveys: Sera of Animals Other than Man

California virus.—California:—In Kern County, California, sera from 3 of 21 cottontail rabbits, 4 of 22 jackrabbits, 11 of 57 ground squirrels, all of 8 horses, and all of 3 cows contained neutralizing antibodies. None of 11 chicken sera had antibodies. A small number of sera from northern California and the State of Washington had no antibodies (4).

Eastern equine virus.—United States:—In 1947 in Louisiana, sera of 9 of 14 horses, 1 of 3 dogs, and 2 of 105 chickens had neutralizing antibodies. No antibodies were found in sera from 1 pig, 8 ducks, 8 geese, 3 turkeys, and 35 pigeons (49). In 1947 in Tennessee, 2 of 31 chickens and 1 of 7 cows had antibodies, whereas 4 horses and 1 dog had no antibodies (57).

Philippines:—Mace *et al.* (58) found that 26 of 86 sera from known un-vaccinated horses in the Manila area had neutralizing antibodies. None of 14 chicken sera had antibodies.

Western equine virus.—Colorado:—In Weld County in 1950, 2.6 per cent of 191 sera from small mammals had antibodies (59). In 1949, 28 per cent of 89 wild and domestic bird sera contained antibodies (60).

Kansas and Missouri:—11.6 per cent of 112 wild bird sera collected in 1949 contained antibodies (60).

Louisiana:—In 1947 the sera of 1 of 3 dogs and 19 of 88 chickens had neutralizing antibodies. The sera from 16 horses, 1 pig, 8 ducks, 8 geese, 3 turkeys, and 30 pigeons had no antibodies (57).

Washington:—Of wild and domestic bird sera collected in the Yakima Valley in 1949, 1 per cent of 302 had antibodies. Comparing the same species, it was found that 24.5 per cent of the sera was positive in 1941 as compared with 1.1 per cent in 1949 (12).

St. Louis virus.—Colorado:—In Weld County in 1949, none of 68 wild and domestic bird sera contained neutralizing antibodies (60). In 1950, 1 of 109 sera from small wild mammals had neutralizing antibodies (59).

Kansas and Missouri:—None of 68 wild bird sera collected in Kansas and Missouri in 1949 had St. Louis antibodies (60). Surveys carried out by Smith and associates (8) in St. Louis County, Missouri, in 1943 and 1944 showed an incidence of 11 to 33 per cent of antibodies in 5 chicken flocks.

Washington:—Of wild and domestic bird sera collected in the Yakima Valley in 1949, 7.7 per cent had neutralizing antibodies. Comparing sera from the same species, 25.8 per cent were positive in 1941 and 9.8 per cent in 1949 (12).

Japanese encephalitis virus.—Japan:—In 1946 at Sapparo there were neutralizing antibodies in the sera of 12 of 30 horses, but in none of 19 cows, 20 swine, and of 5 chickens. At Tokyo there were antibodies in the sera of 14 of 22 horses, 2 of 16 cows, 7 of 20 swine, 2 of 20 goats, and none of 18 chickens. At Okayama there were antibodies in the sera of 9 of 20 cows, 8 of 16 goats, but none of 20 horses and of 15 chickens. At Kumamoto, there were antibodies in the sera of 13 of 20 horses, 3 of 10 swine, and none of 20 cows (51). At Okayama during the summer of 1947, Burns & Matumoto (61) observed 14 horses, initially without antibodies, and found that all acquired neutralizing and complement-fixing antibodies by the end of the summer. In addition, they observed that 5 of 6 cows and 1 of 8 sheep acquired neutralizing antibodies. Various species of animals observed at Yokohama, Saitama Prefecture, and Tokyo did not acquire antibodies. Of 56 wild bird sera collected by Hammon *et al.* (62) in 1949, 7 had neutralizing antibodies. In a later collection, 21 per cent of 214 had antibodies. Large water birds such as herons, egrets, and cormorants, together with magpies, had the highest percentage of positive sera.

Korea:—In 1946 in the vicinity of Seoul all of 20 horse sera, 14 of 20 cow sera, all of 20 swine sera, and none of 11 chicken sera had neutralizing antibodies. Of sera collected at Kunsan and vicinity all of 9 horse sera, 16 of 17 cow sera, all of 10 swine sera, and none of 12 chicken sera had antibodies (52). Following the 1949 epidemic, neutralizing antibodies were found in all of 30 horses sera, all of 17 swine sera, all of 14 dog sera, 20 of 30 cattle sera, 12 of 25 sheep sera, 1 of 5 chicken sera, and none of 3 goat sera (22).

Okinawa:—In 1945 and 1946, 77 of 82 horses sera, 16 of 22 cow sera, 18 of 42 goat sera, 19 of 23 swine sera, 1 of 2 thrush sera, and none of 24 chicken, 4 duck, and 15 crow sera had neutralizing antibodies (28).

Malaya:—Indigenous Malayan ponies from various areas possess neutralizing antibodies (25).

Philippines:—Neutralizing antibodies were found in the sera of 13 of 29 cattle, 10 of 11 carabaos, 2 of 8 goats, and 21 of 23 horses (29). Mitamura *et al.* (27) report that in 1943 25 of 44 horses and 4 of 38 cattle had antibodies.

Other areas:—Antibodies have been found in horse sera from Sumatra, Java, Burma, Formosa, and South China (27).

Murray Valley encephalitis virus.—In 1951, Anderson *et al.* (53) found complement-fixing antibodies in the sera of 37 of 38 horses in northern Victoria, of 4 of 191 in Victoria south of the Divide, of 2 of 7 in Queensland, of 11 of 110 in New South Wales, of none ⁀f 40 in Western Australia, and of 0 of 3 in Tasmania. Sera of 5 of 9 foxes and 2 of 3 opossums from New South Wales had complement-fixing antibodies. In northwestern Victoria 15 of 20 species of water birds and 7 of 20 species of land birds were found to have neutralizing antibodies.

Miles & Howes (55) found that 29 of 86 sera from horses in South Australia and the Northern Territory had neutralizing antibodies, 13 also had complement-fixing antibodies, and one had complement-fixing antibodies alone. Sera collected in April 1952 from 50 domestic fowl and 6 young cormorants in northwestern Victoria did not have neutralizing antibodies according to Anderson (54).

Rift Valley fever virus.—Smithburn *et al.* (56) found no neutralizing antibodies in the sera from 72 monkeys, 1 buffalo, and 1 waterbuck in Bwamba, Uganda.

Yellow fever virus.—Clark (63), from the Gorgas Memorial Laboratory, during 1949 and 1950 collected sera chiefly from monkeys but a few from other species in Panama; 104 of 201 sera collected east of the Canal and 68 of 224 collected west of the Canal had neutralizing antibodies. Early in 1951 monkey sera were collected in southern Mexico in the State of Chiapas. Three of 43 had neutralizing antibodies. Rodaniche (64) from the same laboratory, using sera from live monkeys purchased from trappers from 1949 through 1951, found that none of the sera from 44 monkeys captured west of the Canal had antibodies although 12 of 157 sera from monkeys east of the Canal had antibodies distributed as follows: 6 of 42 in 1949, 2 of 36 in 1950 and 4 of 79 in 1951. A pygmy anteater captured in the Canal Zone during 1951 and 2 agoutis captured in the Province of Darien in 1951 had transitory neutralizing antibodies. In part, according to Rodaniche, the difference between these figures and those of Clark may be explained by the number of juvenile animals in the last series.

Africa:—Dick (65), working in Uganda, found 1 of 9 hyraxes, 1 of 3 Red River hogs, and 2 of 15 mongooses with antibodies. Haddow *et al.* (66) in Uganda between 1942 and 1947 found 43.7 per cent of 1025 primate sera

to have antibodies. The incidence varied from 4.1 per cent in monkeys under six months of age to 67.5 per cent in adults of three years or older. The highest incidence was found in lowland rain forest. Two adjacent similar areas might differ widely with respect to the number of immune monkeys. Rapid appearance of antibodies was seen in Bukasa Island in Lake Victoria, where, in April 1943, 1 of 33 monkeys had antibodies, while in December 1943, 32 of 36 had antibodies. Of monkeys exclusively arboreal, 60.5 per cent had antibodies, of those mainly terrestrial, antibodies were present in 35.2 per cent. In Kenya, Haddow (67) found galagos superior to monkeys as an indicator of the presence of virus; 14 of 81 galagos had antibodies as against 5 of 271 monkeys.

Antibody surveys properly controlled for specificity are very sensitive indicators of virus presence. They usually show that virus is also present during interepidemic and interepizootic periods. They point to animal species which must be considered in the maintenance of virus. When sera are obtained from representative age groups the years of marked dissemination of virus can be determined; this may vary for different species.

ISOLATION OF VIRUS FROM MOSQUITOES

California virus.—Virus was isolated in Kern County, California, from two lots of *Aedes dorsalis*, collected in June 1943 and June 1944, and from one lot of *Culex tarsalis* collected during July 1944. A similar virus has been isolated from *A. trivittatus* collected in North Dakota (4).

Eastern equine virus.—Chamberlain *et al.* (68) isolated virus from *Culiseta melanura* collected August 17, 1950, in southern Louisiana. Howitt *et al.* (69) isolated virus from *Mansonia perturbans* collected in Georgia in the summer of 1948.

Western equine virus.—In Weld County, Colorado, in the summer of 1950, virus was isolated on two occasions from 4492 *A. dorsalis*. No virus was isolated from 1141 *C. tarsalis* (70). Virus was not isolated from 3277 *C. tarsalis* collected in the Yakima Valley in the summer of 1949, in contrast to the findings from 1941 through 1944 when virus was frequently isolated (12).

Japanese encephalitis virus.—China:—The isolation of virus from *C. pipiens* was reported (19).

Japan:—Hammon and associates (28, 71) reported the isolation of virus from nine lots of *C. tritaeniorhynchus* collected in Okayama before the onset of the 1948 epidemic and in 1949 from two lots of *C. tritaeniorhynchus* collected in Tokyo before the appearance of cases. Mitamura *et al.* (72, 73) isolated virus in 1942 at Okayama from mice bitten by three lots of *C. tritaeniorhynchus* and one lot of *C. tritaeniorhynchus* containing two unidentified mosquitoes. In only one instance was there illness in the initial mouse. Isolations in previous years are summarized without giving mosquito species or passage number in mice. In the summer of 1938 virus was isolated from 7 mice exposed to mosquito bites, in the summer of 1939 from 13 mice, in

the summer of 1940 from 2 mice, and in 1941 no isolations were made. Sabin (74) was unable to isolate any virus from 2915 *C. tritaeniorhynchus*, 1410 *C. pipiens*, 1490 *Anopheles hyrcanus sinensis*, and 506 *Armigeres obturbans* collected in various parts of Japan in the summers of 1946 and 1947.

Okinawa:—Mosquito collections were made by various American workers from 1945 through 1948. No virus was isolated (28).

Guam:—No virus was isolated from 20,361 mosquitoes collected from February to March 1948, and from November to January 1949. *A. pandani* and *C. quinquefasciatus* were the predominant mosquitoes (75).

Rift Valley fever virus.—Smithburn et al. (56) isolated virus from two pools of 2307 and 2326 mosquitoes of the *A. tarsalis complex*, three pools of *Eretmapodites* species containing 441, 942, and 347 mosquitoes, respectively, and one pool of 60 *A. de boeri* subspecies *de-Meilloni* collected between April 18 and May 27, 1944, in Bwamba, Uganda. Only the *Eretmapodites* group appeared to contain large amounts of virus.

ISOLATION OF VIRUS FROM MITES

Eastern equine virus.—Virus was isolated from approximately 300 *Dermanyssus gallinae* collected in Tennessee in August 1947 (57).

Western equine virus.—Hammon and associates (76) state that from wild bird mites an isolation is made for approximately every 11,000 mites examined, and isolations do not precede those made from mosquitoes. Virus was isolated by Miles et al. (77) from about 500 *D. americanum* collected in an English sparrow's nest in Weld County, Colorado, July 18, 1950. They examined 78 lots of about 500 mites each. There were 35 lots of *D. americanum*, 23 *D. gallinae*, 5 *Liponyssus sylviarum*, and 15 of various mixtures of these species.

ISOLATION OF VIRUS FROM ANIMALS WITHOUT APPARENT DISEASE

Eastern equine virus.—Virus was isolated by Kissling et al. (78) from the blood of a purple grackle shot in southern Louisiana, June 19, 1950.

Western equine virus.—Sooter and associates (79) collected 1491 blood specimens from 20 species of nestling birds in Weld County, Colorado, in 1950, 151 in June, 454 in July, 501 in August, and 257 in September. Virus was isolated from 2 of 55 blood specimens from red-winged blackbirds and from 1 of 224 from magpies. These specimens were collected between June 26 and June 30.

LABORATORY STUDIES ON VIRUS TRANSMISSION

Isolation of virus from a mosquito or mite species must be confirmed by transmission studies before it can be concluded that they play a role in a virus cycle. Demonstration of antibodies in, or isolation of virus from the blood of an animal must be confirmed by the demonstration of viremia of sufficient extent to infect a mosquito or mite.

Studies with mosquitoes.—California virus:—Reeves & Hammon (80) in

limited studies had a successful transmission with *A. dorsalis* after feeding on a mouse brain virus suspension diluted 1 to 1000.

Eastern equine encephalitis virus:—Chamberlain has secured transmission with *Psorophora ferox, P. confinnis, P. ciliata*, and *P. howardii* (42).

Venezuelan encephalitis virus:—Levi-Castillo (18) reports transmission by *C. quinquefasciatus* and *A. taeniorhynchus*.

Japanese encephalitic virus:—Chao & Chung (19) state that Huang found *A. chemulpoensis* to transmit the virus readily to mice but *C. pipiens* did not. Hammon and associates (81) transmitted virus to mice by the bite of *C. tritaeniorhynchus* and *C. pipiens*. Hurlbut & Thomas (82) reported transmission by *C. annulirostris* and *C. quinquefasciatus* to mice. According to Kitaoka *et al*. (83), seven species. *C. tritaeniorhynchus, C. pipiens, Aedes japonicus, A. togoi, A. albopictus, Armigeres obturbans*, and *Anopheles hyrcanus sinensis* can transmit the virus to mice. Tigertt & Hammon (28) state that American workers in 1949 confirmed transmission by *A. togoi* and *A. albopictus*.

Rift Valley fever virus:—Smithburn *et al*. (84) showed that two lots predominately *Eretmapodites chrysogaster* after feeding on infected mice or lambs, could transmit virus to these species of animals.

Studies with mites.—Reeves (85) was unable to repeat the work on mite transmission of St. Louis virus by Smith *et al*. (86) and also had negative results with the western and eastern equine viruses. He states that Chamberlain also had negative results.

Viremia in Experimental Animals

California virus.—Virus was present in undiluted blood of ground squirrels 48 to 60 hr. after subcutaneous inoculation of 100 LD_{50} of virus (87).

Western equine encephalitis virus.—Hammon and associates (76) found, after inoculation of small amounts of virus subcutaneously, that English sparrows had viremia from the first to the fourth day after inoculation with a maximum titer of 10^4 on the first day, that the house finch had viremia as long as the ninth day with a titer of 10^4 during the second and third days, that the tricolored red-winged blackbirds had a titer of 10^6 on the second day and a small amount of virus as long as the tenth day, and that the white crowned sparrow had a titer of 10^5 on the second day and some virus as long as the eleventh day.

St. Louis encephalitis virus.—The same species of birds had viremia of about equal duration but of much lower titer except in white crowned sparrows which had a titer of $10^{4.5}$ on the third day. On the third day, the house finch had a titer of $10^{2.3}$ and the tri-colored red-winged blackbird a titer of $10^{1.3}$. In the English sparrow virus was detected in undiluted blood (76).

Japanese encephalitis virus.—A low grade viremia was found in chickens and English sparrows. In house finches virus was found from the first to the seventh day, with virus in the 10^{-2} dilution on the second and third days.

In the tricolored red-winged blackbird virus was found from the first day to the sixth day and in the 10^{-3} dilution on the third day (62).

Murray Valley encephalitis virus.—Miles (88), using large amounts of virus intracerebrally, found virus in undiluted blood for a few days in young chickens, pigeons, and domestic ducks, and in silver gulls up to eight days.

Rift Valley fever virus.—Smithburn *et al.* (84, 89) in two lambs found virus on the second, third, and fourth days following bites of infected mosquitoes. The maximum amount of virus found was $10^{4.7}$ LD_{50} per ml. on the third day. In accidental infections in laboratory personnel, over 10^6 LD_{50} per ml. was found on the first and second days of illness. Virus was found from the first through the fifth day of illness.

Yellow fever virus.—In hedgehogs obtained in Kenya, Dick (65) found a titer of $10^{4.5}$ on the fourth day following subcutaneous inoculation. Three of six genets tested had viremia. The maximum amount of virus found in one animal was $10^{2.4}$ on the sixth day.

Virus cycles.—To support laboratory and field observations pointing to certain vectors and their hosts as of importance in maintaining a virus, attempts are occasionally made to set up such cycles in the laboratory. Ross & Gillett (90) maintained yellow fever virus through three complete cycles using Grivet monkeys and *A. africanus.* Hurlbut & Thomas (91) carried Japanese encephalitis virus through one complete cycle using *C. quinquefasciatus* and 3 to 12-day-old mice.

ECOLOGICAL STUDIES OF SUSPECTED VIRUS CYCLES

Western equine virus.—Jenkins (92) collected data on the distribution of *C. tarsalis* and showed that its distribution fits the known distribution of the equine disease quite well.

Japanese encephalitis virus.—China:—At Peking the breeding season of *A. chemulpoensis* is July and August and fits the occurrence of human disease better than that of *C. pipiens* which extends from early May to late October.

Japan:—Mitamura *et al.* (72, 73) collected mosquitoes within buildings at the Okayama Medical School throughout 1941 and 1942. The peak population of *C. tritaeniorhynchus* appeared to fit the occurrence of encephalitis best. In 1941 *C. tritaeniorhynchus* had its peak population early in August when it comprised one-third of all mosquitoes collected. Similar conditions were present in 1939 and 1940. In 1942 there was a sharp peak in the middle of July, and during late July and early August it comprised 92 per cent of mosquitoes collected. A similar condition was found in 1938.

During the summer of 1946 in miscellaneous collections Sasa & Sabin (93) found *C. tritaeniorhynchus* dominant in Okayama City with *C. pipiens* and *Anopheles hyrcanus sinensis* present. In mountain districts where cases had been reported *C. vishnui* was dominant with *A. hyrcanus sinensis* present. *C. tritaeniorhynchus* was absent, and *C. pipiens* practically so. In a

lowland coastal area *C. tritaeniorhynchus* was dominant and *Mansonia uniformis* well represented. *C. tritaeniorhynchus* fed predominantly on domestic mammals, while *C. pipiens* fed more frequently on domestic birds. *A. hyrcanus sinensis* fed on domestic mammals and not on chickens. Sasa and associates (94) using baited traps in Tokyo found *C. pipiens* and *C. tritaeniorhynchus* to be the dominant species in 1948, and *C. tritaeniorhynchus* and *Aedes vexans* in 1949 using different collection sites. *C. tritaeniorhynchus* appeared suddenly at the end of July and toward the end of August disappeared rapidly. *A. hyrcanus sinensis* had a population curve similar to *C. tritaeniorhynchus*. They found the same feeding preferences as did Sasa & Sabin. Kitaoka and associates (83) also collected mosquitoes in Tokyo and vicinity during the years 1948 and 1949, chiefly from domestic animal shelters. The four most commonly collected species were *C. tritaeniorhynchus*, *A. hyrcanus sinensis*, *A. vexans*, and *C. pipiens*. From July to September *C. tritaeniorhynchus* was the predominant mosquito. *C. tritaeniorhynchus* was the mosquito most frequently collected in domestic animal traps. *C. pipiens* attacked man more frequently.

Okinawa:—Tigertt & Hammon (28) have summarized mosquito collections by various investigators on Okinawa in 1945, 1946, 1947, and 1948. *C. quinquefasciatus*, *C. tritaeniorhynchus*, and *A. hyrcanus sinensis* were the common species found.

Guam:—Reeves & Rudnick (75) made a survey in Guam in February and March 1948, following an encephalitis epidemic, and again from November 1948 to January 1949. In human dwellings 99 per cent of mosquitoes collected were *C. quinquefasciatus*. In human bait collections at the margins of native villages, *A. pandani* was the dominant mosquito. Using stable-type traps, *A. pandani*, *A. vexans*, *A. subpictus*, *C. annulirostris*, and *C. quinquefasciatus* were the common species collected. It was concluded that *C. quinquefasciatus*, *C. annulirostris*, *A. vexans*, *A. albopictus*, and *A. pandani* should be considered as possible vectors.

Murray Valley encephalitis virus.—Burnet (95) considers that *C. annulirostris* is almost certainly the principal vector. In the Murray Valley it is a river-bottom mosquito, feeding freely on water birds and rabbits.

Rift Valley fever virus.—In enzootic and epidemic areas Gear *et al.* (33) found *C. theileri* abundant. *Aedes caballus* was next in abundance. Both species are nondomestic but do feed on sheep and man. They state that *Eretmapodites chrysogaster* is not found in the Union of South Africa. Smithburn *et al.* (84) state that it is improbable that *E. chrysogaster* could propagate in the open country in Kenya where the disease occurred in the past. It is also a daytime feeder, but evidence from Kenya was that a night biting insect was involved.

Yellow fever virus.—Africa:—In Uganda, transmission of yellow fever virus to human beings has been associated with the presence of *A. simpsoni*. Gillett (96) has found that in Uganda there are biting and nonbiting populations of *A. simpsoni*, as far as human beings are concerned. He attributes

the relative absence of yellow fever antibodies in the human population in Uganda outside of Bwamba to the presence of the nonbiting population. In Kenya, the lemurs, *Galagos crassicaudatus* and *G. senegalensis*, appear to be more important than monkeys in maintaining yellow fever virus. According to Haddow (67) lemurs are active during the night and sleep in nests during the day and do not appear to be good mosquito hosts.

SURVIVAL OF VIRUS

Many writers recently have pointed out the difficulty of accounting for survival of virus during periods unfavorable for mosquitoes. Sabin (74) and Sasa & Sabin (93) question that the few overwintering adult *C. tritaeniorhynchus* or *A. hyrcanus sinensis* could maintain the Japanese encephalitis virus in Japan. Galindo *et al.* (97) point out that during the dry season in Panama all the *Haemagogus* species virtually disappear together with *A. leucocelaenus*, and they raise the problem of survival during this period. Taylor & Theiler (98) point out that in southern Brazil it is not known how yellow fever virus survives through the cold season when known mosquito vectors virtually disappear. To Ross & Gillett (90) it appears that in some small isolated forests in Uganda where the immune proportion of monkeys is high and where the dry season is relatively prolonged, the primate-mosquito cycle does not seem adequate to account for the maintenance of virus. Haddow *et al.* (66) suggest the possibility that in yellow fever the monkey-to-monkey cycle, like the man-to-man cycle, may be the end point of a chain about which nothing is known at present.

Transovarial transmission.—Japanese encephalitis virus:—During 1941 Mitamura & Kitaoka (72) could not confirm their previous work indicating transovarial transmission in *C. pipiens.*

Yellow fever virus:—Gillett *et al.* (99) were unable to obtain any evidence of transovarial transmission in *A. africanus.*

Carry-over in hibernating mosquitoes.—Japanese encephalitis virus:— Hurlbut (100) reported that infected *C. quinquefasciatus* kept at 8° to 13°C. for 82 days to simulate natural hibernation were still capable of transmission. In discussing isolation of virus from mosquitoes, Mitamura *et al.* (73) emphasize that isolations were obtained only during the second half of July, August, and the first half of September, although collections were made throughout the year.

Western equine and St. Louis encephalitis viruses:—From January 8 to the middle of May 1951, bi-weekly inspections of cellars were made in Scottsbluff County, western Nebraska by Keener (101). The lowest temperature encountered in the cellars was 33°F. *C. tarsalis* was found at nearly every visit. The largest number caught at any visit was 15. Up to March 3, 47 were caught in one cellar, and 24 in another. Eighteen mosquitoes were tested for virus with negative results.

Carry-over in auxiliary cycles involving mites or ticks.—Since viruses of diseases such as louping ill, Russian spring summer encephalitis, and Nairobi

sheep disease are transmitted by and carried over from one season to the next
in ticks, it has long been suggested that a similar mechanism may be found
to account for the survival of mosquito-transmitted viruses over unfavorable
seasons. Smith and associates (8) reported that St. Louis virus had main-
tained itself in a *Dermanyssus gallinae* mite colony for three years and that
it was possible to produce viremia in chickens by feeding these mites on
them. Mosquitoes feeding on these chickens later were able to transmit St.
Louis virus to other chickens. Viremia in all the chickens had to be demon-
strated by two serial passages on the chorio-allantoic membrane of chick
embryos and then by inoculation of mice. Direct inoculation of mice pro-
duced no illness. Mites could not be infected with a California strain of St.
Louis virus. As mentioned under transmission studies, other workers have
not been able to transmit virus with mites. Rather large numbers of ticks
of various species have been examined at the Rocky Mountain Laboratory
without isolating any mosquito transmitted virus.

Periodic reintroduction of virus from more favorable areas.—Migrating
birds:—The hypothesis that migrating birds transport virus apparently was
first presented by Ten Broeck (102) several years ago in connection with his
studies of eastern equine encephalomyelitis. It has been stressed recently by
Australian workers in connection with their study of Murray Valley en-
cephalitis. Burnet (95) postulates that persistence of virus in nature is con-
fined to tropical or near tropical areas, where mosquitoes capable of trans-
mitting virus are present the year around. Migrating birds then transport
the virus through regions in which effective mosquito transmitters are pres-
ent. Foci of infected mosquitoes are built up which then infect other birds,
which, in turn, carry the virus farther and start new foci. No evidence of
such spread has yet been presented.

Transportation of infected mosquitoes by air currents.—Haddow et al. (66,
in discussing the problem of maintenance of yellow fever virus in small
forests and in dry areas, suggests the possibility that infected mosquitoes are
introduced periodically from favorable areas by convection currents and
winds.

Other mechanisms.—Sasa & Sabin (93) mention the possibility that an
intestinal parasite or other parasite of domestic animals maintains the virus
by transmission from one generation to the next. Foster & Shahan (103)
could not isolate virus from parasites collected from moribund horses follow-
ing experimental acute encephalitis (whether eastern or western not stated).
Findlay and Howard (104) were able to demonstrate the presence of Rift
Valley fever virus in the larvae of *Taenia crassicolis* removed from the liver
of mice inoculated with this virus.

VIRUS RELATIONSHIPS

Serological relationships have been pointed out among members of the
mosquito-transmitted viruses. Such relationships are of interest because
they may indicate a common origin and similarities in ecology.

California virus.—Hammon *et al.* (87) found that St. Louis and Japanese encephalitis virus antigens showed slight fixation in the presence of California virus immune serum.

Murray Valley virus.—French (105), Miles & Howes (55), and Pond *et al.* (106), using the complement-fixation and neutralization tests, found the Murray Valley virus related to the Japanese, St. Louis, and West Nile viruses, with closest relationship to the Japanese virus.

Japanese virus group and dengue and yellow fever viruses.—With the use of hyperimmune sera against dengue virus, yellow fever virus, and Japanese encephalitis virus, and the complement-fixation test, Sabin (107) was able to show that there was a common antigen present in low titer in the dengue, yellow fever, Japanese, and West Nile viruses. He showed interference between yellow fever virus and dengue virus in human volunteers, and, jointly with Theiler, in rhesus monkeys and *Aedes aegypti* (108, 109). The relationship between dengue and yellow fever viruses and members of the St. Louis-Japanese group may possibly point to a common origin. The significance of the serological relationship of the West Nile virus to these viruses is not clear because so little is known of its natural history.

Other relationships.—Smithburn (110) did cross-neutralization tests with the nine viruses isolated by yellow fever workers in Africa and South America from mosquitoes, and with the well-known mosquito and tick transmitted viruses. He found many antisera neutralizing heterologous viruses; such neutralization was generally unilateral and only slight in amount. At present the interrelations found by Smithburn do not make an understandable pattern, but they do suggest that arthropod transmitted viruses may be shown to fall into definite groups.

Newly Described Viruses

Ntaya virus was isolated by Smithburn & Haddow (111) in February 1943 in Uganda by the inoculation of mice with a suspension of 1318 mosquitoes, consisting of approximately 24 species, with *Culex* species predominating. No antibodies were found in the sera of 43 people living near the Ntaya swamp where the mosquitoes were collected. Zika virus was isolated by Dick *et al.* (112) in mice by the inoculation of a suspension of 86 *A. africanus* collected in the Zika Forest, Uganda, and, in April, 1947, from the blood of a sentinel monkey. Antibodies were found in 4 of 71 people living in Uganda. Uganda S. virus was isolated by Dick & Haddow (113) from a lot of 65 mosquitoes consisting of three *Aedes* species, predominantly *A. longipalpis*, by the inoculation of mice. Antibodies were found in 5.8 per cent of 121 sera from Uganda residents and in 1 of 9 monkey sera. Smithburn (114) used these three viruses and five other African viruses in neutralization tests with 297 sera from residents of Uganda and Tanganyika. 14.5 per cent neutralized the Ntaya virus, 12.8 per cent the Zika virus, and 7.7 per cent the Uganda S. virus. It appeared that a person who acquired immunity to either Ntaya, Zika, Uganda S., or West Nile virus had a much greater than normal

chance of acquiring immunity to another of this group, indicating, perhaps, transmission by similar vectors. During the past decade five new viruses have been isolated from mosquitoes in Africa and four in South America by yellow fever workers (110). Their isolation is of great significance in emphasizing the importance of arthropods in the dissemination of viruses.

An Estimate of Progress

The large amount of study devoted to these viruses has yielded meager results as measured by the establishment of specific mosquito-vertebrate cycles.

It was established relatively quickly and easily that the cyclic passage of yellow fever and dengue viruses between man and certain domestic mosquito species was a very efficient mechanism for maintaining these viruses. When in the study of yellow fever it became necessary to search for virus cycles among wild animal populations the difficulties of study were increased greatly because of the number of animal species that had to be investigated. Investigators with very different scientific backgrounds, working cooperatively, were necessary to establish the cyclic transfer of virus between certain tree hole breeding mosquitoes and primates.

Other mosquito transmitted viruses have been studied much less intensively. Only in the case of the western equine virus is a mosquito-vertebrate cycle adequately established. Here a bird-*Culex tarsalis* cycle appears to be important. Evidence for this cycle in the case of the St. Louis virus is less convincing. In Japan the importance of *C. tritaeniorhynchus* in disseminating the Japanese virus is indicated by the isolation of virus from this species, positive transmission experiments, good correlation of peak mosquito population with the occurrence of human disease, and by its feeding habits. The vertebrate part of the cycle has not been established. Although man and domestic animals have a high incidence of antibodies, it is not known whether they are at the end of a cycle or are part of a continuous virus cycle. With the remainder of the viruses in this group the existence of mosquito cycles is indicated chiefly by the epidemiological pattern of the disease caused by the virus, or by the isolation of virus from mosquitoes, or by both criteria.

With respect to all of the viruses which occur in the temperate zones, evidence of the presence of virus has been obtained only during a few summer months. The real or apparent absence of virus for most of the year has led to speculation concerning other mechanisms for its maintenance during this period. Although many alternative mechanisms to the mosquito-vertebrate cycle have been proposed, none can be considered as having been established. The conditions under which a mosquito-vertebrate cycle fails to account for the maintenance of virus must be much better understood before there can be an intelligent search for another mechanism. Present knowledge of the mosquito-vertebrate cycle does not even permit a definite statement that an alternative cycle is necessary.

Studies to date are quite adequate to demonstrate the importance of mosquitoes in disseminating virus.

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ADRENOCORTICAL HORMONES IN INFECTION AND IMMUNITY[1]

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The impact of the discovery that cortisone (11-dehydro-17-hydroxy-corticosterone, or compound E) and pituitary adrenocorticotropic hormone (corticotropin, or ACTH) alter the clinical course of rheumatoid arthritis (1) has made itself felt in almost every field of medicine (2). Clinical observations were rapidly extended to include a wide variety of inflammatory disorders, and it was soon found that these hormones alter the clinical course in many diseases. It thus became clear that basic physiologic mechanisms involved in many diseases of quite different etiologies were affected by these hormones. Most of the diseases in which ACTH or cortisone exert a clinical effect are entities without known or completely acceptable etiology, pathogenesis, or experimental laboratory model; progress towards an understanding of the mechanisms by which these hormones exert their effects has, therefore, been slow. When studies of the effects of these hormones were extended to selected infectious diseases, in which etiology, pathogenesis, and host responses could be more clearly defined, it became possible to formulate some of the effects of the cortical hormones in terms of known mechanisms. It is the purpose of this review to summarize some of the available data in terms of the effects of these hormones on resistance to infection and to indicate how such data may contribute to our understanding of basic mechanisms of resistance and of the mechanisms of action of the adrenal cortical steroids in clinical conditions in which they have been used. Reviews of various aspects of this subject are already available (2–6).

Some Aspects of the Normal Physiologic Function of the Adrenal Cortex

T   secretions of the adrenal cortex are not indispensable for life, but they are essential for the capacity of the body to readjust its economy to a steady state in response to marked alterations in its external or internal environment. Thus, almost any type of noxious stimulus to which laboratory animals are subjected will, if sufficiently prolonged or severe, cause hypertrophy of the adrenal cortices, indicating that animals undergoing stress produce larger amounts of cortical hormone than resting animals (7).

The varied degrees of responsiveness of the adrenal cortex suggest that

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[1] The survey of literature pertaining to this review was completed in December, 1952. It was intended primarily as a critical review; limitations of space prevented a complete summary of the pertinent literature.

there may be no fixed rate of secretion of hormone by the adrenal cortex and presumably, no fixed rate at which peripheral utilization proceeds (8, 9). Indeed, doses of adrenal cortical steroids which are necessary to maintain an animal undergoing a severe stress may be so much greater than the needs of a resting animal that they may induce evidence of hyperadrenalism when injected into the latter (9). This suggests that the production of adrenal steroids may be linked to the rate of peripheral utilization so that the syndrome of hypercorticalism is not seen in acute stress, despite clear evidence of increased production of adrenocortical hormones. Certain physiologic changes which accompany such severe stresses as infections and fevers may occur in the absence of adrenal cortical secretions (10, 11). Although other physiologic changes require the presence of such hormones, it is by no means clear that the increases in output of hormones are responsible for the changes that occur (8, 9, 12, 13). Instead, the concept has arisen that adrenal cortical steroids mediate certain physiologic patterns that are characteristic of the response to many noxious stimuli, but are not quantitatively linked to them (8, 9, 14, 15, 16).

Although little is known about peripheral utilization of adrenal hormones, it may be surmised that they regulate some aspects of metabolism in almost every cell of the body (8). Hardly an organ system has failed to manifest some evidence of disturbance when adrenal cortical hormones were withdrawn, and there is ample evidence of a regulatory role of these hormones on metabolism of carbohydrate, fat, protein, electrolytes, and a wide variety of specific substances (7, 8, 13). Indeed, the number of physiological activities which can be shown to be affected by the adrenocortical hormones seems to be limited primarily by the number of investigators with special interests who have sought to elicit them. This multiplicity of effects suggests that different tissues and cells may each respond in special ways to the same hormones depending on the particular functions of the cells concerned and upon their functional state at the time. The adrenocortical hormones must, therefore, regulate metabolic activities at levels that are so fundamental as to be common to many cells and to many activities within the cells. There is at present no reason to believe that these hormones initiate new reactions.

The rate of secretion of steroids by the adrenal gland is under the immediate control of the hypophysis (9), and the production of corticotropin by the hypophysis is under control of at least two factors: (a) a hypothalamic one which may be humoral or neural (17 to 20), and (b) the level of adrenal steroids or some metabolic derivatives in the circulating blood (8, 9, 13). Thus, the administration of sufficient adrenocortical steroid induces atrophy of the adrenal cortex in part, at least, by depressing endogenous production of ACTH (9). Present evidence suggests that only one adrenocorticotropic hormone is produced by the hypophysis, and this hormone acts only on the adrenal cortex (8). The interrelationships among the endocrine glands are complex, and it is frequently difficult to distinguish a direct effect of a hormone from the indirect effects of the same hormone on other glands with

consequent disturbances of seemingly unrelated functions (8, 13).

The precise composition of the adrenal cortical secretion is still uncertain. Recent evidence suggests that in certain animal species, such as oxen, the adrenal glands secrete corticosterone (compound B of Kendall) and hydrocortisone (17-hydrocorticosterone, or compound F of Kendall) (21). Continued administration of ACTH leads to increasing production of steroid, with relatively more hydrocortisone than corticosterone being produced. The available evidence suggests that the predominant steroid secreted by the adrenal of man is hydrocortisone (22, 23), whereas in the rat and the rabbit it may be corticosterone (24). Hydrocortisone and cortisone are clinically active in rheumatoid arthritis in man, but corticosterone is not (25, 26, 27). Species variations in steroid production have not been sufficiently appreciated in the literature. Moreover, the possibility cannot be dismissed that minute amounts of highly active secretory products have been overlooked. One such substance, a steroid, has recently been isolated from adrenal vein blood and shown to have much greater salt-retaining activity than desoxycorticosterone (28). Cortisone does not appear to be a predominant product of the adrenal cortex.

The "adaptation syndrome" has been advanced as a unifying concept in this general field. This syndrome has been described as consisting of three stages: the alarm reaction, the stage of resistance, and the stage of exhaustion (29). In essence, it has been suggested that when a stress is continued after the acute reaction to it has occurred, anatomic and physiologic changes may take place, and these have been linked to "exhaustion" of the capacity of the hypophyseal-adrenal system to respond optimally. Under such circumstances widespread gross and histologic changes in many organs may appear and give rise to many common diseases which are therefore considered to be "diseases of adaptation." The validity of this provocative thesis is under investigation in many laboratories. The pertinence of questions of adaptability to stress to the study of resistance to infectious disease is apparent. More detailed presentations of the views presented here are contained in reviews previously cited (8, 9, 13).

ADRENOCORTICAL HORMONES IN RELATION TO INFECTIOUS DISEASE

The need to study the effects of adrenocortical hormones in diseases of relatively defined etiology and pathogenesis led to investigations in patients with pneumococcal and viral pneumonias (30). Additional observations have now been reported in such diverse infections as tuberculosis (31 to 33), brucellosis (34), typhoid fever (35, 36), subacute bacterial endocarditis (37), other bacterial pneumonias (38, 39), Rocky Mountain spotted fever (40, 41), leptospirosis (42), tetanus (43, 44), poliomyelitis (45, 46), viral hepatitis (47, 48, 49), acute streptococcosis (50), malaria (51, 52), trichinosis (53 to 57), blastomycocis (58), peritonitis (59), smallpox (60), mumps (61), and, undoubtedly, others. Although the etiologic agents or the specific serologic responses were not clearly defined in all cases, the following

generalizations seem warranted from the available data: (a) Administration of ACTH or cortisone to patients with acute febrile illnesses or with illnesses characterized by malaise, anorexia, and evidence of generalized toxicity frequently leads to prompt defervescence, and amelioration of these symptoms; (b) in infections with less prominent symptoms these effects of the hormones are less striking, or may be absent; (c) despite the relatively asymptomatic and afebrile state that follows administration of adrenocortical hormones, bacteriologic findings indicate no apparent improvement or even definite impairment in the capacity of the patient to dispose of the offending agent. Bacteremia may develop or persist, bacterial counts in exudates or affected tissues may rise, and the lesions may spread even in the absence of clinical evidence of such adverse effects; (d) antibody production is neither accelerated nor suppressed with doses employed clinically; (e) complications of the infections may occur, although their presence may be masked by the administration of ACTH or cortisone; (f) cutaneous hypersensitivity reactions are frequently diminished or suppressed; (g) in poliomyelitis (45) and streptococcosis (50), which are frequently mild diseases clinically but which may have serious sequelae, controlled studies show that the duration of illness and the incidence of sequelae are not significantly different in cortisone-treated as compared with control patients.

Evaluation of the effects of adrenal steroids in certain infections is especially difficult. For example, in viral hepatitis, there may be decreased bilirubinemia and improvement in the malaise and anorexia (47, 48). The decrease in serum bilirubin may reflect a direct effect on hepatic inflammation, may be due to increased intake of food (a common nonspecific effect of these hormones), or may result from some other relatively nonspecific effect on hepatic or renal clearance of bilirubin.

The effect of simultaneous administration of specific antibiotics and ACTH or cortisone has been studied in experimental animals but has not been investigated in a systematic or controlled manner in human infections. The few pertinent data that are available suggest that, as in the case of infections treated with cortisone or ACTH alone, there is prompt amelioration of many of the symptoms but no evidence of potentiation of the effect of the antibiotic (33, 35, 39 to 44, 59). It has not always been possible to identify adverse effects from the clinical studies, because of the nonspecific feeling of well-being induced by the hormones, but studies in experimental animals, to be summarized below, indicate that a sufficiently large dose of ACTH or cortisone may interfere with optimal activity of antibiotics.

Table I presents a summary of the pertinent results of studies of infections in laboratory animals. A few deductions and comments appear warranted:

(a) Cortisone and ACTH usually depress resistance of laboratory animals to a wide variety of bacterial, viral, protozoal, and fungal agents. Although cortisone has consistently depressed resistance, and ACTH has done so in rabbits, rats, rhesus monkeys, and probably also in guinea pigs, the latter

failed to depress resistance of mice to infections, even when used in doses equivalent to 10 International units every 12 hr. (69, 70). Similarly, the resistance of hamsters to the virus of poliomyelitis was not altered by ACTH, although cortisone was effective in depressing resistance (119, 120). A noticeable effect on resistance to infection has been observed in only one group of experiments in mice; in them large doses of ACTH were administered in an adjuvant to prolong its action (100). Unfortunately, only four mice were given the hormone alone to determine if this dose of hormone was of itself sufficient to cause death of the mice. Highly purified corticotropic hormone, suspended in oil and beeswax and given to the limit of tolerance, did not significantly depress resistance to influenza viral or pneumococcal infections in partially protected mice (70). The dose of ACTH used caused more lymphopenia and weight loss than was produced by doses of cortisone which depressed resistance to the same infections (69, 70). This suggests that ACTH causes the hematologic and weight changes through means other than adrenocortical stimulation, or that it induces secretion of one or more hormones which are qualitatively and quantitatively different from cortisone, hydrocortisone, or corticosterone, all of which depress resistance to infection in the mouse. The adrenal glands of mice manifest only minimal morphologic changes after the administration of ACTH (132), and hamsters may also react atypically. Further studies are needed to determine the significance of these peculiar species differences.

(b) ACTH or cortisone may activate latent infections or render animals susceptible to fatal infection by inhabitants of the respiratory or intestinal tracts which ordinarily are nonpathogenic (64, 65, 66, 94).

(c) Depression of resistance occurs regardless of whether the resistance is native, as in the case of strains of rabbits that are genetically resistant to tuberculosis (82, 83), or acquired, as in the case of mice given partially protective doses of specific antiserum (69, 70) or of vaccine (80). However, in experimental systems employing animals with no effective immunity to the infectious agent, such as guinea pigs with tuberculosis, or unimmunized mice with pneumococcal infections, it may not be possible to demonstrate aggravating effects of any treatment because susceptibility is already maximal and the infection is usually fatal even after the introduction of only one to three organisms.

(d) Effective doses of ACTH or cortisone are usually associated with evidence of increased multiplication of the pathogen, more widespread dissemination, and diminished local inflammatory responses. Although adrenal cortical hormones are not stimulatory to bacterial growth *in vitro* (133), cortisone caused greater multiplication of certain viruses in embryonated eggs (112, 113), in which immunity has not been demonstrated. However, in tissue cultures, cortisone did not alter viral multiplication (134).

(e) Infected animals receiving cortisone or ACTH may succumb to smaller doses of infective agent then do animals not receiving these hormones. The doses of hormone used by different workers are not shown in

TABLE I

Effects of ACTH and Cortisone on Experimental Infections

Infectious Agent	Animal	Hormone	Effect of Hormone	Ref.
BACTERIA				
Brucella abortus	mice		Increased multiplication of organisms; converted mild into fatal infection in mice; chronic disease unaffected.	62
Brucella melitensis	guinea pigs	cortisone		
Brucella suis	rabbits			
Brucella abortus	guinea pigs	ACTH	Enhanced infection.	63
Corynebacterium kutscheri				
(*C. pseudotuberculosis*	mice	cortisone	Induced spontaneous and often fatal infections by non-pathogens.	64
murium)	rats			65
Coliform organisms	mice	cortisone	Induced spontaneous infections.	66
Diplococcus pneumoniae	rabbits	cortisone	Bacteremia and mortality increased.	67
Diplococcus pneumoniae	rats	cortisone	Shortened survival; decreased pulmonary inflammation.	68
Diplococcus pneumoniae	mice	ACTH	Little effect.	69
	mice	purified ACTH		70
Diplococcus pneumoniae	mice	cortisone hydrocortisone corticosterone	Earlier deaths, greater susceptibility, even in partially immunized mice.	69, 70
Diplococcus pneumoniae	rabbits	cortisone	Infection aggravated, penicillin effect reduced.	71
Diplococcus pneumoniae	mice mice	adrenal cortical extract	No effect on recovery in severe, penicillin-treated infections. Sulfonamides not potentiated.	72 73
Diplococcus pneumoniae	rabbits	cortisone	Increased bacteremia.	74
Mycobacterium tuberculosis	guinea pigs rats	cortisone	Enhanced infection; streptomycin effect reduced.	75, 76, 77 78
Mycobacterium tuberculosis	guinea pigs & mice	cortisone	Increased numbers of bacilli in organs.	79
Mycobacterium tuberculosis	mice	cortisone	Infections and mortality enhanced.	80
Mycobacterium tuberculosis	guinea pigs	cortisone, ACTH	No observed effect.	81
Mycobacterium tuberculosis	rabbits	cortisone, ACTH	Depressed resistance in genetically resistant animals.	82, 83
Mycobacterium tuberculosis	rats	cortisone	Infection and mortality enhanced.	84
Mycobacterium tuberculosis	rabbits	ACTH	Inconsistent effect on sensitized and treated animals.	85
Mycobacterium tuberculosis	mice	cortisone	Overcame beneficial effect of vaccine.	86
Mycobacterium tuberculosis	mice	ACTH	Shorter survival, streptomycin therapy unaffected.	140
Mycobacterium tuberculosis	rabbits	ACTH, cortisone, hydrocortisone	Adverse effect on ocular lesion.	87
Mycobacterium tuberculosis	rabbits	ACTH, cortisone	Infection aggravated inconstantly.	88
Neisseria meningitidis	mice	cortisone	No effect on mortality.	89
Pasteurella muricida	rats	cortisone	Converted asymptomatic carrier state to fatal infection.	90
Pleuropneumonia-like organisms	rats	cortisone, ACTH	Polyarthritis not prevented.	91, 92
Salmonella typhosa	rabbits	cortisone	Susceptibility increased; slowed clearing of organisms.	138
Staphylococcus aureus	rabbits	cortisone	?Protected against toxicity.	67
Staphylococcus aureus	rabbits	cortisone	Skin lesions aggravated, healing retarded.	93
Streptococcus faecalis	mice	cortisone	Produced spontaneous infection.	66
Streptococcus faecalis	mice	cortisone	Produced spontaneous infection and aggravated induced infection.	94
Streptococcus pyogenes (*hemolyticus*)	mice	cortisone	Lymphadenitis aggravated.	68
Streptococcus pyogenes (*hemolyticus*)	rats	cortisone	Pretreatment aggravated infection despite penicillin. Concomitant treatment with penicillin beneficial.	68, 95
Streptococcus pyogenes (*hemolyticus*)	rabbits	cortisone, ACTH	Increased susceptibility.	96
Streptococcus viridans	mice	cortisone	Increased incidence and mortality of arthritis.	97
Treponema pallidum	rabbits	ACTH	Delayed repair; less inflammation.	98
Treponema pallidum	rabbits	cortisone	Increased numbers of spirochetes; lesion altered; reagin decreased.	99
VIRUSES				
Anopheles B	mice	cortisone	No effect.	100
Bunyamwera	mice	cortisone	Diminished resistance.	100
Coxsackie	mice	cortisone	Rendered adult mice susceptible.	101, 102, 103
Encephalitis, Japanese B	mice	cortisone	Depressed resistance.	104
Encephalomyelitis, Western equine	mice	ACTH	No effect.	105

TABLE I—(*continued*)

Infectious Agent	Animal	Hormone	Effect of Hormone	Ref.
Encephalomyocarditis	mice	cortisone	More rapidly fatal infection early.	101
Herpes simplex	rabbits	ACTH, cortisone	Severity of early keratitis retarded.	106
Herpes simplex	rabbits	cortisone	Diminished corneal reaction; prolonged infection; delayed healing.	107
Herpes simplex	rabbits	cortisone	Keratitis aggravated.	108
Ilheus	mice	ACTH	No effect.	100
Ilheus	mice	cortisone	Increased susceptibility.	100
Infectious fibroma	rabbits	cortisone	Larger tumors, if given with virus; no effect if started later.	109
Infectious myxoma	rabbits	cortisone	Mortality unaffected, symptoms reduced.	109
Influenza A	ferrets	ACTH	No effect.	110
Influenza A	mice	ACTH, cortisone	?Decreased viral multiplication.	111
Influenza A	mice	ACTH, purified ACTH	No effect.	70
Influenza A	mice	cortisone, hydrocortisone	Increased and hastened mortality; viral multiplication not altered.	69, 70
Influenza A	rats	cortisone	Increased virus in lung.	70
Influenza A & B	embryonated eggs	cortisone	Increased viral titers.	112, 113
Influenza A	mice	ACTH	No effect.	114
Mumps	embryonated eggs	cortisone	Increased viral titers.	112
Pneumonitis virus of mice	mice	ACTH	Susceptibility unaffected; ?viral multiplication enhanced.	115
Pneumonitis virus of mice	mice	cortisone	Enhanced infection and viral titers.	115
Poliomyelitis	monkeys	ACTH	?Paralysis hastened.	116
Poliomyelitis	mice	ACTH, cortisone	No effect.	116
Poliomyelitis	mice	cortisone, ACTH	Higher viral titers, earlier deaths.	101
Poliomyelitis	hamster	cortisone	Enhanced infection.	101
Poliomyelitis	monkeys	ACTH	Increased incidence of paralysis slightly.	117
Poliomyelitis	mice	ACTH	No effect.	105
Poliomyelitis	hamsters	cortisone	No effect.	118
Poliomyelitis	mice	cortisone	Increased susceptibility in suckling	118
Poliomyelitis	monkeys	cortisone	Exacerbated disease.	118
Poliomyelitis	mice	ACTH	No effect.	
Poliomyelitis	mice	cortisone	Decreased resistance.	119, 120,
Poliomyelitis	hamsters	ACTH	No effect.	121
Poliomyelitis	hamsters	cortisone	Decreased resistance.	
Poliomyelitis	mice	cortisone	Enhanced infection.	103
Rift Valley Fever	mice	cortisone	Higher viral titers, earlier deaths.	101
Vaccinia	rabbits	cortisone	Inflammation decreased; virus decreased and disseminated.	139
Vaccinia	guinea pigs	cortisone	Skin lesions aggravated, healing delayed.	93
West Nile	mice	ACTH	Large doses enhanced infection.	100
West Nile	mice	cortisone	Depressed resistance.	100
West Nile	mice	DOCA	No effect.	100
FUNGI				
Achorion quinckeanum	guinea pigs	cortisone ACTH	Prolonged primary infection.	122
Blastomyces dermatitidis	mice	cortisone	Enhanced infection.	103
Candida albicans	mice	cortisone	Enhanced infection.	103
Trichophyton mentagrophytes	guinea pigs	cortisone	Skin lesions larger, more persistent.	93
PROTOZOA				
Coccidioides immitis	rats	cortisone	Enhanced infection.	90, 123
Coccidioides immitis	rats	DOCA 21-acetoxypregnenalone	No effect.	123
Plasmodium berghei	rats	cortisone	Increased parasitemia and mortality; induced relapses.	124
Plasmodium cynamolgi	monkeys	cortisone	Prolonged parasitemia post-crisis; induced relapses.	125
Plasmodium knowlesi	monkeys	cortisone	Increased incidence of blackwater water.	126
Plasmodium relictum	pigeons	cortisone	Increased parasitemia; induced relapses.	127
Trypanosoma cruzi	dogs	ACTH	Improved clinically; organisms unaffected.	128
Trypanosoma cruzi	mice	cortisone	Increased parasitemia.	129
Trypansoma cruzi	mice	cortisone	Increased virulence.	130
Trypanosoma vickersae	monkeys	cortisone	Induced relapses in latent infection.	131
HELMINTHS				
Trichinella spiralis	guinea pigs	ACTH	Slightly increased survival.	54

the table, but they have varied widely and were seldom chosen on the basis of the amounts needed to induce specific biologic effects in the recipient animal. For example, adult mice of one strain required 1.25 to 2.5 units of corticotropin to produce more sustained eosinopenia and lymphopenia than that produced by the handling of the animals and administration of non-hormonal proteins (135); yet such large doses have not always been used in the experiments listed in the table. Undoubtedly as long acting and more purified preparations of ACTH become available it should be possible to decrease the dose of hormone; thus, a highly purified preparation of corticotropin, when administered to mice in oil and beeswax, had about 50 times the activity by weight of the original Armour 1A-LA standard (70). Cortisone has generally been available in such forms that the relatively small amounts that have been used have frequently been adequate to produce biologic effects. Nevertheless, there is evidence that small laboratory animals may require relatively large amounts to produce metabolic or hematologic evidence of hyperadrenalism (69).

Although the hyperadrenal state appears to be a necessary concomitant of effective concentrations of ACTH or cortisone, a dose of hormone which will induce hyperadrenalism in the intact uninfected animal may not be sufficient to do so in the animal undergoing stress. Thus, the adrenalectomized mouse, partially protected with antiserum, succumbs to approximately one-tenth as many pneumococci as the intact mouse similarly protected. The amount of cortisone that will restore the adrenalectomized animals to approximately the original state of resistance to infection is 5 to 10 times the amount which will apparently maintain the uninfected adrenalectomized mouse. Approximately 20 to 50 times the minimal maintenance dose must be given to the adrenalectomized mouse to depress resistance beyond the level which is maintained in the intact animal (136). It is apparent that host differences and the state of availability of the hormone are important variables, as is the state of the endocrine balance at the time the infection and the hormones are introduced.

(f) When ACTH or cortisone is given in conjunction with antibiotics, the hormones generally reduce the effectiveness of a given dose of antibiotics. It is, nevertheless, possible, in acute experimental infections, to give sufficiently large doses of antibiotics to overcome this decreased effectiveness (68, 71, 72, 73, 95). In chronic processes, such as tuberculosis, cortisone may reduce the localizing effect and fibrous tissue reaction that occur when a given dose of streptomycin is given to the infected animals (75, 76, 77); it is not clear, however, whether larger amounts of the antibiotic would overcome this effect.

(g) In few instances was there evidence that the infected animal with intact adrenals is benefited by these hormones (54, 67, 128). In one series of experiments, evidence was advanced that the survival of rabbits infected heavily with Staphylococcus was enhanced by cortisone (67). However, the animals did not have staphylococcal bacteremia at death. These results

will be discussed in the section dealing with the effects of cortical hormones on toxicity.

(h) Resistance has been decreased in experimental animals by the use of these hormones as a means for facilitating the isolation of infectious agents (101, 102, 103, 137). This procedure has not always been successful (124), however, and it is not clear that the method will lead to the more rapid adaptation of pathogens to the intact animal. The dangers of using such methods for the isolation of infectious agents that are difficult to identify is apparent; spontaneous or latent infections may be exacerbated and give rise to pathologic changes that may be misleading.

Clinical counterparts of most of these experimental findings have been observed, and many have already been enumerated. In addition, such infections as pneumonia (141, 142), bacteremia due to various organisms (143, 144, 145), pyelonephritis (146), peritonitis (147, 148), diffuse moniliasis (149), other mycoses (150), tuberculosis (151 to 159), and others (143, 160, 161, 162) have occurred during the course of therapy with ACTH or cortisone. Inasmuch as the clinical manifestations of these infections have frequently been altered by the administration of the cortical hormones, such infections have occasionally been unrecognized and have been discovered only at autopsy. The appearance of infection during the course of treatment with ACTH or cortisone has been one of the major reasons for temporary or permanent cessation of hormonal therapy (163). However, not all of the reported cases represent activation of latent infection or appearance of new infection. In many instances, patients with fever of unknown origin were treated for "collagen disease" although the diagnosis of tuberculosis or other infection was not clearly ruled out. Under such circumstances, deaths due to tuberculosis might fairly be ascribed to the ordinary progression of unrecognized tuberculosis, possibly accelerated, in some instances, by the administration of adrenocortical hormones. Many warnings have been issued to physicians urging that the use of adrenal cortical hormones in patients with possible latent tuberculosis be avoided and that these hormones be used with circumspection where there is the possibility that infection exists or may occur (3, 158, 162, 164, 165).

As was previously mentioned, clinically effective doses of ACTH or cortisone almost invariably induce metabolic and anatomic changes characteristic of hyperadrenalism. It is of interest that in spontaneous hyperadrenalism (Cushing's syndrome) infection is the leading cause of death, accounting for over half the deaths in a large series studied, and the incidence of infection as a cause of death has not decreased significantly since the advent of modern chemotherapy (166).

Effect of ACTH and Cortisone on Toxicity

Inasmuch as no beneficial action of adrenocortical hormones on experimental infections has been demonstrable, it has been thought possible that the apparently favorable clinical effects of these hormones may be referable

to some interference with the toxic action of bacterial products on host metabolism. However, ACTH did not increase the survival time or the mortality rate in mice or rats receiving acute toxic doses of influenza virus (167), nor did the hormone seem to benefit mice injected with toxic doses of Rickettsiae (167, 168), or *S. typhosa* endotoxin (168). Cortisone did not protect mice against the effects of *Shigella* endotoxin (89). Similarly neither ACTH nor cortisone protected guinea pigs against lethal doses of diphtherial toxin (89, 169). In one series of observations in which cortisone-treated guinea pigs were given large doses of toxin intraperitoneally, it was observed that early deaths were delayed, but the ultimate mortality of the animals was the same as in controls (170). The adrenal cortices of cortisone-treated guinea pigs failed to show the hemorrhagic reaction that is usually seen after the administration of lethal doses of diphtherial toxin (89, 169).

Intradermal injection of a single dose of meningococcal toxin, intended as a preparatory dose in eliciting the Shwartzman phenomenon, was followed by early erythema and edema in control animals; the reaction in cortisone-treated animals was characterized by the absence of edema but by the development of a hemorrhagic skin lesion that was indistinguishable from that of the complete Shwartzman phenomenon (171). The intravenous injection of a single dose of toxin from meningococci or from *Sarcina marcescens* into cortisone-treated rabbits resulted in hemorrhage and necrosis in the kidneys and in other viscera of rabbits comparable to that seen when control animals were given two injections of toxin a day apart (172). Thus, cortisone apparently may replace the preparatory injection used to elicit either the localized or generalized Shwartzman phenomenon (172, 173). The resistance of the rabbit to otherwise mildly toxic doses of toxin was evidently diminished as a consequence of administration of cortisone. Even a single intradermal injection of toxin into cortisone-treated rabbits has led to the development of renal necrosis (173), whereas ordinarily the toxins that induce the Shwartzman phenomenon are not rapidly absorbed from an intradermal site of deposition, and the provocative injection must usually be made into the vein. These observations suggest that cortisone interferes with the capacity of skin tissue to localize, or to detoxify toxin injected into it.

On the other hand, it has been claimed that cortisone protects mice against toxic derivatives of *Brucella* organisms (34). It has already been noted that when cortisone was given to rabbits injected with large numbers of staphylococci, fewer hormone-treated than control animals died, and that those animals which received cortisone and died did not have bacteremia, suggesting that a toxic action of the staphylococci was overcome (67). Similar experiments also suggest that the administration of cortisone to rabbits may protect them to some degree against toxic doses of typhoid vaccine (67).

Although the weight of evidence suggests that ACTH and cortisone do not confer increased protection to animals challenged with toxins of bac-

terial or viral origin, there is no general agreement. The numbers of observations are too small to permit analysis in terms of species differences, types of toxins employed, duration of action, etc. This important area warrants more intensive study, particularly because the possibility has often been expressed that the cortical hormones may assist the host in some manner to resist the toxic effects of infectious agents. Such a statement is not completely supported by the evidence at hand.

Many of the toxins that have been used in the aforementioned studies are also pyrogens, when injected in sublethal concentrations. The administration of ACTH or cortisone to animals receiving pyrogens generally results in reduction of the fever. Such antipyretic action of these hormones has been observed using as pyrogens typhoid bacilli in man (174), or typhoid bacilli (67, 174), bacterial extracts (175, 176, 177), or influenza virus in rabbits (174 to 178), and it has been observed that ACTH is actually hypothermic (177) and may reduce basal body temperatures in the resting animal by 1 to 3 degrees. In addition, ACTH hastens the resolution of fever if given at the height of the response to a pyrogen (177). An antipyretic action of ACTH or cortisone has not uniformly been demonstrated (176, 178); dosage may play a critical role, and with relatively small doses of hormone the febrile response may actually be accentuated rather than suppressed (176). The explanation for the latter phenomenon is not clear. However, the hypothermia and suppression of fever [in one series of experiments ACTH compared favorably in the latter respect with large doses of acetylsalicylic acid (174)] suggest that nonspecific suppression of fever may account for many of the apparently beneficial effects of the cortical hormones in a variety of febrile disorders.

Three possible mechanisms by which antipyretic activity could take place may be mentioned: (a) there may be a direct effect on hypothalamic heat-regulating centers, (b) there may be increased peripheral heat loss, or (c) there may be accelerated removal or detoxification of the pyrogen. The first is a likely explanation of the hypothermia, and hence of some of the antipyretic activity. Profuse diaphoresis may accompany defervescence clinically after the administration of ACTH or cortisone, hence the peripheral mechanism may be activated. The third mechanism may be the basis for the "tolerance" to pyrogens that occurs in man or in experimental animals. When animals have become tolerant to a pyrogen, the injection of a blockading agent such as India ink may restore susceptibility to the fever-producing activity of the pyrogen, apparently by interfering with the removal of the pyrogen by the reticulo-endothelial cells (179). Thus, it is conceivable that ACTH or cortisone may be antipyretic, in part, by accelerating the rate of removal of toxin from the circulation. However, although many of the toxins used in these experiments are pyrogens, it cannot be assumed that the antipyretic action of ACTH or cortisone is a reflection of protective action against the toxic pyrogen. It is not established that tolerance to the pyretic effects of a toxin carries with it tolerance to the lethal

effects. Furthermore, hypothermia is demonstrable in the animal treated with ACTH alone (177), hence some effect on the temperature-regulating mechanism must occur independently of an effect on toxins. Finally, as has been shown earlier, there is little evidence that capacity to resist toxins is enhanced by the administration of adrenocortical hormones.

Effect of ACTH and Cortisone on Reactions of Immunity and Hypersensitivity

Circulating antibody.—Despite earlier indications to the contrary (180, 181, 182), there is little reason to believe that ACTH or cortisone augments antibody production or release. With the use of quantitative immunochemical methods it was shown that large amounts of adrenal steroids cause inhibition of the antibody response in rabbits (183, 184, 185), rats (186), mice (187), and guinea pigs (188). Although cortisone appears to be more effective as a supressing agent, the difference between the two may be related to dosage or mode of administration, inasmuch as long-acting preparations of ACTH were not used. In man, inhibition of antibody formation has not usually been observed (189 to 192) but the relative degree of hypercorticoidism was probably not so great as in experimental animals.

The rate of disappearance of passively administered antibody is not altered by the administration of adrenocortical hormones (193), suggesting that the hormones interfere with synthesis of antibody. The observation that cortisone interferes with the rapid increase of antibody titers in the anamnestic response (194) supports this concept. Finally, cortisone diminished the degree of response of pentose nucleic acids (PNA) in regional lymph nodes to the injection of antigens (195). Such increases in PNA have been associated with antibody formation, and cortisone not only diminished the PNA concentrations in unstimulated lymph nodes, but also altered the normal pattern of response of PNA to the presence of antigens (196, 197). The conclusion seems warranted that ACTH and cortisone interfere with the synthesis of antibody. Such a conclusion is consistent with evidence derived from clinical balance studies which indicate that cortisone is "anti-anabolic" with respect to general body stores of protein (198), and it receives support from other observations which indicate that incorporation of amino acids into body protein is hindered after the administration of cortisone (199, 199a). The latter contention, however, is controversial (200 to 202).

Mechanisms other than inhibition of synthesis may be involved in the suppression of the antibody response. For example, local antibody production and the local granuloma that occur when adjuvants are used have been related to infiltration of large mononuclear cells (203, 204). Inasmuch as local cellular responses to antigens are inhibited by the administration of cortisone or ACTH, antibody formation may be impaired. This may explain why cortisone protects against experimentally produced disseminated encephalomyelitis in which the characteristic formation of local granulomata is also inhibited by cortisone (204).

When autologous or heterologous erythrocytes are injected subcutaneously into cortisone-treated rabbits, such cells persist within the macrophages of the regional lymph nodes for several days after the lymph nodes of control animals show no further evidence of the injected erythrocytes (195, 205). Similar evidence of delayed breakdown and removal of foreign or native materials has been obtained using labeled proteins (205); there is also evidence of persistence, and even proliferation, of bacteria within macrophages in cortisone-treated animals, in contrast to the rapid disappearance of bacteria in control animals (83). Perhaps the effects of cortisone on the synthesis of antibody, on the local inflammatory response, and on the capacity of phagocytic cells to break down or dispose of ingested materials are related to a common mechanism, but the evidence for such an hypothesis is still inadequate.

The clinical effects of the administration of ACTH or cortisone are probably not related to an effect on antibodies because (a) clinically effective doses of the hormones do not seem to alter antibody formation greatly, and (b) the rate of decline of preformed antibody after administration of cortisone to animals is not sufficiently rapid to account for the clinical changes which so frequently occur within the first day after treatment is started.

Hypersensitivity reactions.—The effects of ACTH and cortisone on many varieties of hypersensitivity reactions have been studied; only a cursory review is possible here. More detailed reviews are available (5, 206, 207).

There is fairly general agreement that neither ACTH nor cortisone affects active, passive, reversed or Forssman-type anaphylaxis in guinea pigs (208 to 215), although there is one conflicting report (216). The active Arthus phenomenon has been inhibited by ACTH or cortisone in rabbits (184), but this appears to be associated with suppression of antibody response, for when the same animals that failed to manifest active Arthus responses were given antibody passively, the adrenal hormones did not alter their reactivity. There is evidence to suggest that the failure of cortical hormones to alter the active development of anaphylaxis in guinea pigs is due, at least in part, to the fact that anaphylaxis requires but small amounts of antibody in the guinea pig (217) and that this animal's antibody response is not sufficiently suppressed by cortisone (188). So great is the apparent resistance of guinea pigs to these hormones with respect to sensitization phenomena that as much as 200 mg./kg. of cortisone, or even more of ACTH did not affect the development of sensitization to dinitrochlorobenzene (218). These studies indicate that in man (219, 220, 221), rabbits (184), guinea pigs (188), or mice (222) ACTH and cortisone do not interfere with the actual union of antigen and antibody.

Cutaneous sensitivity to allergens is not usually diminished by therapy with ACTH or cortisone in patients who have experienced marked clinical relief from manifestations of hypersensitivity (219, 221). Neither the release of histamine nor the effects of injecting histamine into man or experimental animals is altered by the use of adrenocortical hormones (208, 211, 212, 214,

223, 224, 225). Likewise, the "immediate" type of hypersensitivity response is not affected.

On the other hand, cortisone and ACTH reduce the inflammatory vascular lesions in experimental serum sickness of the rabbit (226 to 236), and cortisone inhibits the anaphylactic raction of the actively sensitized mouse (237). From the latter experiments, however, it is not clear whether there was any effect on antibody production since titrations either were not done or were performed with methods that may not accurately reflect the content of antibody nitrogen in serum. In contrast to these effects on active sensitization, the nephrotoxic nephritis that is produced by passive transfer of appropriate anti-kidney sera is not affected by adrenal hormones (238, 239).

It is apparent that cortical hormones do not readily alter those cutaneous reactions which develop rapidly and are associated with the release of histamine-like materials and with reactions that are primarily exudative rather than proliferative. They do, however, affect delayed cutaneous reactions that are characterized by cellular infiltrations. Thus, tuberculin-type hypersensitivity is frequently, though inconstantly, inhibited in man as well as in experimental animals; tuberculin, Frei antigen, and streptococcal products are among the antigens that have been studied (240 to 245). That the effect is not on hypersensitivity *per se* is indicated by the observation that mononuclear cells obtained from cortisone-treated animals are still able to transfer tuberculin sensitivity to tuberculin-negative recipients (246). Similar observations have been made using the Prausnitz-Küstner reaction (247). Cortisone is effective locally to some degree (248), although hydrocortisone appears to be more effective (249) under such circumstances.

Effect of ACTH and Cortisone on Inflammation and Repair

Immunologically active substances are not the only ones to which the inflammatory response may be altered by adrenocortical hormones. Cortisone, hydrocortisone, and ACTH may also inhibit cellular inflammatory reactions to a wide variety of other irritants including burns, trauma, jequirity seed, formaldehyde, Croton oil, talc, turpentine, and others (241, 250 to 255).

The precise components of the inflammatory response that are inhibited by adrenocortical hormones cannot be clearly defined at present. The initial, transient vasoconstrictive stage of inflammation, which may represent a local reflex, has not been adequately studied. The subsequent stage of vascular reactivity to an irritant is distictly modified (226, 256). Capillary and arteriolar tone is increased, permeability to plasma proteins is diminished, endothelial swelling is reduced, and adherence and clumping of the particulate elements of blood are virtually eliminated. The extent and cellular content of the exudate are thereby reduced, although necrosis is not necessarily averted. Although phagocytosis by polymorphonuclear leukocytes may be diminished somewhat (257), there may be increased phagocytic

activity by large mononuclear phagocytes (253). The numbers of small and large mononuclear cells in the exudate are usually markedly reduced (253, 258).

Reparative processes, including new capillary formation and fibrogenesis, may also be inhibited (259 to 266). The extent to which constituents of connective tissue, such as ground substance, collagen, or other chemical substances, are specifically affected by adrenal cortical hormones is still only a matter of inference. Because hyaluronic acid and related mucopolysaccharides are important constituents of ground substance, the effect of cortisone on hyaluronidase has received much attention. The accelerated spreading of dyes and the increased permeability of membranes that occur in the presence of hyaluronidase may be overcome by adrenal cortical steroids (227, 267 to 272), but many other substances that have limited, if any, anti-inflammatory activity may also do this (227, 268, 270, 272, 273). However, cortisone and adrenal cortical extracts inhibit the spreading of dyes in the absence of hyaluronidase (274, 275, 276), and hyaluronidase is not inhibited by cortisone *in vitro* (277). Direct measurements of tissue resistance to injection of fluids indicate that this resistance is increased by cortisone and decreased by hyaluronidase, but that each acts independently of the other (275). To what extent the increased tissue resistance that occurs after the administration of cortisone is related to changes in capillary permeability is not clear. It has been demonstrated that the spreading response of skin may be divided into an early and a late phase; only the early phase is directly controlled by hyaluronidase while the late phase is related to the accumulation of edema. Cortisone does not affect the early phase of spreading, but reduces edema and may thus inhibit spreading (277).

Studies of other substances such as hyaluronidase inhibitors (278, 279, 280), serum mucoproteins (278, 281) and C-reactive protein (282) have also been made in patients undergoing treatment with cortisone or ACTH. Alterations in the serum concentrations of these substances may be related to changes in some feature of the inflammatory response, but no specific relationship between any of these factors and cortisone activity has yet been demonstrated.

The reported adverse effects of adrenal steroids on wound healing and the favorable clinical effects in many diseases which are characterized by deposition of connective tissue, have prompted investigations of the effects of these hormones on connective tissue reactions. Although inhibition of fibrogenesis has been demonstrated in many ways (259 to 266), the usual difficulties of interpretation related to species differences and to dosage have arisen. From the clinical point of view, there is much individual variation, as might be anticipated, but delayed healing and diminished fibrogenesis have occurred inconstantly in surgical and other types of trauma and suppression of granulation tissue has been more conspicuous than alteration of fibrogenesis in primary healing (283). Probably this effect on granulation

tissue explains the occurrence of perforated intestinal ulcerations and similar complications of treatment with ACTH and cortisone (147, 148).

DIRECT EFFECTS OF ACTH AND CORTISONE ON CELLS

It is commonly assumed that diminished fibrogenesis represents a direct effect of the adrenal hormones on connective tissue elements; the evidence, however, is not completely secure. It is not clear to what extent the altered sequence of events preceding fibrogenesis is responsible for the later inhibition of the reaction. It is conceivable, for example, that the failure of cellular exudate to appear in the inflammatory site may lead to a deficiency of some fibroblast-stimulating factor (284).

Studies of tissue cultures have not yielded definitive results. Growth of fibroblasts was reported to be inhibited and lymphocytic migration and survival to be diminished (287) by small amounts of cortisone (285, 286), but there were great variations from culture to culture. Some controlled studies indicate that there may be no effect of cortisone on fibroblasts in tissue culture unless huge amounts of cortisone are present (288, 289). The important observation that incorporation of radioactive sulfate into organically bound forms is inhibited in cultures containing cortisone (290) requires further confirmation in view of other studies which indicate that cortisone does not affect the uptake of radio-phosphate into nucleic acid fractions, and that it has no significant effect on concentrations of nucleic acid in suspended cell cultures of allantoic sacs of chick embryos (134).

The rate of lysis of lymphocytes *in vitro* has been reported to be hastened by adrenal steroids in some experiments (291) but not affected in others (292). Extracts of lymph nodes are required to demonstrate lysis of lymphocytes consistently; however, even with such extracts, cortisone and corticosterone fail to affect lysis, whereas adrenocortical extracts accelerate it (293). Adrenal cortical extracts and cortisone retard the growth of chick embryos, but so does desoxycorticosterone (294, 295, 296). Cortisone in high concentrations alters the permeability of echinoderm eggs, but other steroids that are not anti-inflammatory have even greater effects (297). It is difficult to harmonize all of these conflicting observations. Cortisone may act selectively on certain tissues, such as fibroblasts, more than on others, such as monocytes and epithelial cells in tissue cultures (285, 298), although as already stated, there is some doubt that such effects are regularly reproducible. It is of interest that epithelialization has not been adversely affected *in vivo* by ordinary doses of ACTH or cortisone (32, 299), although very large doses or prolonged administration may lead to some changes in the epidermis (300, 301). At present, the evidence that cortisone directly alters connective tissue metabolism *in vitro* is not secure; this very important problem requires further investigation.

Many features of the anti-inflammatory action of ACTH and cortisone remain unexplained. It is not clear, for example, why the passive Arthus phenomenon should be unaffected (184) by the action of adrenocortical

hormones whereas the Shwartzman phenomenon, which is histologically similar (302), is inhibited (216); nor is it clear why, in the Shwartzman phenomenon, the effects of the preparatory dose of toxin should induce an unusually severe response in the cortisone-treated animal (173), whereas the effects of the second, or challenge dose of toxin may be inhibited (216, 303, 304).

MECHANISMS INVOLVED IN DECREASED RESISTANCE TO INFECTION

A summary of the possible mechanisms whereby excesses of ACTH or cortisone may depress resistance to infection is given in Table II. The depression of inflammation and the effect on antibody formation have been discussed. The role of induced negative nitrogen balance is difficult to assess. Protein deficiency may accentuate susceptibility to infection in many diseases in which the effects of adrenocortical steroids have been studied (305). However, in certain viral and protozoal diseases, available evidence

TABLE II

MECHANISMS BY WHICH ACTH OR CORTISONE MAY DEPRESS RESISTANCE TO INFECTION

I. Inhibition of inflammation
    A. Decreased capillary permeability
    B. Decreased cellular exudation and infiltration
    C. Decreased exudation of fluid and protein
    D. Decreased phagocytosis by polymorphonuclear leukocytes
    E. Decreased fibrogenesis and repair
II. Inhibition of antibody production
III. Negative nitrogen balance
IV. Alteration of reticulo-endothelial function

suggests that nutritional deficiencies, if they alter susceptibility at all, actually lessen it (305). The possibility that specific nutrients which are critical to the multiplication of certain pathogens may be mobilized by the hormones has not been ruled out. It is to be recalled also that in mice, ACTH, in doses which induced more weight loss than did the doses of cortisone that were used, failed to depress resistance to pneumococcal and influenza viral infections, whereas cortisone consistently did so (69, 70).

Many observations suggest that reticulo-endothelial function is disturbed by excessive concentrations of adrenocortical steroids: (a) the effects on capillary permeability may represent alteration of normal function of the endothelium, (b) the adverse effect of adrenal steroids on malaria, in which antibody and the acute inflammatory exudate are not prominent as defensive mechanisms, suggests an effect on reticulo-endothelial function; (c) cortisone apparently inhibits the capacity of the reticulo-endothelial system to fix or to remove bacterial toxins from tissues (173).

Although the phagocytic function of large macrophages is probably not inhibited, and may actually be accelerated (253), the capacity of such cells to dispose of the ingested materials seems to be inhibited (205). Exudates containing large macrophages are more readily inhibited by cortisone than are exudates composed primarily of polymorphonuclear leukocytes (258), and detailed study of the histologic effect of cortisone on inflammation indicates that local proliferation was less affected than cellular infiltration, particularly of the mononuclears (244). There is conflicting evidence with respect to the effect of cortisone on removal of bacteria from the blood stream, in that tubercle bacilli are removed more rapidly (83) and pneumococci more slowly (74) under the influence of large doses of cortisone. However, the slowed removal of pneumococci may be related to a special property of the pneumococcus since the removal of particulate dyes in cortisone-treated rabbits is not accelerated (306, 307). The tendency of many types of particles, whether antigenic or not, to be retained locally (205) may in part result from suppression of edema and local exudation with consequent limitation of the movement of particles into lymphatic channels, but this may also be the result of functional changes in macrophagic cells.

RELATIONSHIP OF GROWTH HORMONE TO ACTH AND CORTISONE

There is ample evidence that large doses of adrenal steroids cause alterations in function in endocrine glands other than the adrenal cortex or hypophysis (7, 8, 29). There are also indications that the metabolic effects of growth hormone may overcome some of the effects of adrenal hormones and of stress (308, 309). The effects of growth hormone on infection and particularly on the effects of cortisone have, therefore, been studied. Rats given ACTH or cortisone develop spontaneous pulmonary infections which are often fatal; the incidence of these fatal infections was reduced when growth hormone was given in addition (310). Detailed studies of the etiology of these infections were not made. In other experiments, however, large doses of growth hormone failed to overcome the adverse effects of cortisone on pneumococcal and influenza viral infections of mice (70). Similarly, the effects of cortisone on induced pneumococcal infections in rabbits were not diminished by the administration of growth hormone (311). Whether these discrepancies are species differences or are results of differences in the type of infectious agent is not clear. Additional evidence of interrelationships among adrenal steroids is indicated by the observation that the simultaneous administration of cortisone with adrenal cortical extracts, which contain many steroids, suppressed the enhancing action of cortisone on experimental tuberculosis in mice (312).

RELATIONSHIP OF ADRENALECTOMY TO ACTH AND CORTISONE

In general, adrenalectomy has been found to diminish the capacity of the body to withstand such stresses as infection, and a more or less complete

return to previous resistance may be achieved by the administration of sufficient amounts of adrenocortical hormone (3, 313). The value of adrenocortical hormones to the patient with Addison's disease hardly requires comment. However, the situation which is presented in most clinical states that are treated with adrenocortical hormones is quite different. In the presence of intact adrenal glands with various levels of functional activity, the doses of ACTH or cortisone that have been required for clinical effect have been those which produce some clinical or metabolic evidence of hypercorticoidism. Such excessive doses may produce effects on other endocrine glands and bodily functions which are of quite a different nature from those observed when insufficiency of adrenal steroids is corrected to normal levels. The effect of ACTH and cortisone in depressing resistance to infection has, therefore, been regarded as pharmacological and not as physiological (8, 9).

SUMMARY AND COMMENT

This review presents a selected summary of the vast amount of data that have accumulated in this field. Clinical implications could only be touched upon. Several principles, however, warrant reiteration. Obviously the hazards of infection during the use of adrenal steroids are large, and clinical awareness of the hazard is essential. Of the many related steroids that have been studied, only cortisone and hydrocortisone are anti-inflammatory in man; perhaps corticosterone may act in a similar manner in certain experimental animals. The actions of these hormones in relation to resistance to infection appear to be pharmacologic ones and are not necessarily related to the actions of these same hormones in the treatment of adrenal insufficiency. The anti-inflammatory action of these hormones is the metabolic action which most nearly parallels their clinical activity, and this suggests that a common effect may account for the depression of mechanisms of resistance and for the clinical effects of these hormones in infections and, perhaps, in other types of diseases as well.

It is noteworthy that in virtually all cases in which ACTH and cortisone alter specific immune mechanisms, it has been possible to demonstrate that an underlying nonspecific mechanism is altered; thus, it is not so much that adrenal steroids alter specific hypersensitivity reactions, as that they alter inflammatory responses of diverse etiologies. Acquired immunity is depressed, but probably because native mechanisms of immunity, which are essential to the effectiveness of acquired immunity, are inhibited. Antibody formation is inhibited, but so is protein synthesis of a broader type.

The value of adrenocortical hormones as experimental tools in the study of fundamentals of nonacquired immunity is apparent. A final question that remains unanswered can be posed thus: If inhibition of inflammation is generally harmful in diseases of known etiology, is it not necessary to examine examine carefully the premise that such inhibition is beneficial in diseases of unknown etiology?

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THE IMMUNOLOGICAL RESPONSE

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In the present review an attempt is made to report on progress in our understanding of the chemical, i.e., molecular processes which are the basis of immunological phenomena. This chemical interpretation of immunological reactions forms also the topic of several reviews (1 to 4a) and books (5, 6).

Antigenicity

Previous work, based on Landsteiner's classical experiments, had shown that antigens of a definite specificity can be prepared by coupling proteins with certain molecules such as diazotized aromatic amino acids, or phenyl isocyanates, and that the specificity of these conjugated antigens is determined by polar groups of the protein-bound hapten. Conjugated antigens can also be produced by coupling proteins with azides (7) or oxazolones (8). The antigenicity of these conjugated antigens depends not only on the nature of their haptens, but also on the number of hapten groups per protein molecule and on the nature of the protein carrier (9). While the azo-phenyl-arsonate group coupled to ovalbumin or the serum proteins is highly antigenic and determinant, it does not elicit antibodies when coupled to tobacco mosaic virus (10).

Some of the haptens combine with proteins not only *in vitro*, but also in the injected organism, thus giving rise to the *in vivo* formation of antigens and, secondarily, of antibodies. Haptens of this type have been called pro-antigens (11); they comprise chiefly aromatic nitro compounds such as 2,4-dinitrophenyl fluoride, chloride, bromide, or sulfonate (12). In contrast to these active dinitrophenyl derivatives, 2,4-dinitrobenzene, 2,4-dinitrophenol, 2,4-dinitrotoluene, and 2,4-dinitroaniline are neither able to combine with proteins nor to elicit antibody formation (12). In view of these results, reports on the antigenic action of glycine, acetate, ethylamine (13) which do not react with proteins, and of salts such as LiCl, $MgCl_2$ (14) are surprising; the assumption that these substances are antigenic is based on the increase in viscosity of the blood serum on addition of the "antigen" (13, 14).

The antigenic blood group substances form the subject of extensive reviews (15, 16), so that they will not be discussed here. The reasons for the antigenicity of natural proteins and for the nonantigenicity of others are not clear. Thus bovine and porcine cytochrome are antigenic for rabbits, whereas equine cytochrome is not antigenic (17). Gelatin acquires antigenicity by coupling with the carbobenzoxy derivative of tyrosine; the immune serum gives precipitates with carbobenzoxy-tyrosyl-serum globulin, but not with carbobenzoxy-tyrosyl-gelatin (7). Myosin from rabbit muscle is antigenic for dogs, but not for rabbits, while actin from rabbit muscle induces the forma-

tion of isoantibodies in rabbits (18). The formation of isoantibodies against kidney tissue is considered as one of the causes of glomerulonephritis (19); glomeruli contain an antigen which elicits nephrotoxic antisera (20). Isoantibodies seem also to be formed when tumor tissue in rats is strangulated by means of a tourniquet; the animals become resistant to the subsequent implantation of viable tumor material (21).

The antigenicity of proteins can be considerably increased by the use of adjuvants (22), for instance, by mixing the antigen with paraffin oil and killed mycobacteria, or with alumina cream (23). Quantitative analyses show that smaller doses of antigen can be used and that the ratio of antibody formed to antigen injected is much larger when adjuvants are used (23). The action of the mycobacteria consists of the production of local granulomas and of hyperplasia of the regional lymph nodes and the spleen (24).

The Fate of the Antigen in the Organism

Preparation of isotopically labeled antigens.—The determination of protein antigens in the injected organism is rendered possible by using antigens which are labeled by radioactive isotopes; the antigen can be determined by measuring the radioactivity of organs, tissues, or their subcellular fractions. Labeling has been accomplished by treatment of proteins with traces of radioactive iodine (25, 26) or by coupling with traces of diazotized sulfanilic acid containing S^{35} (27, 28) or anthranilic acid (*o*-amino-benzoic acid) containing C^{14} in its carboxyl group (29).

If labeling is carried out by coupling proteins with only traces of radioactive material, the trace-labeled protein retains its serological specificity; thus, antibodies against bovine serum albumin do not distinguish between untreated and lightly iodinated serum albumin (25, 30) or serum globulin (26), provided that the number of iodine atoms per protein molecule is not higher than two or three. This process of labeling proteins with small amounts of radioactive isotopes must be distinguished from the well known coupling of proteins with large amounts of iodine or of diazo compounds; if radioactive isotopes are used in the latter procedure, trace-labeled iodoproteins and azoproteins are obtained (27, 29, 31).

It is confusing that the designations iodoprotein or azoprotein have been used for both the lightly labeled natural proteins and the heavily substituted iodoproteins and azoproteins. In the present review the serologically unchanged proteins will be designated by their original names, the heavily substituted proteins by adding the name of the substituent. The radioactive element, which in both types of proteins is present in traces only, will be added after the name of the antigen; thus beef serum albumin-I^{131} or BSA-I^{131} means beef serum albumin labeled by traces of radio-iodine, whereas iodo-beef serum albumin-I^{131} or iodo-BSA-I^{131} refers to heavily iodinated beef serum albumin. While BSA-I^{131} elicits the formation of antibodies which combine with BSA, injection of iodo-BSA-I^{131} induces the formation of antibodies which react with iodo-BSA, iodo-ovalbumin, and other heavily iodi-

nated proteins, but not with unsubstituted BSA. It is important for the interpretation of experimental results that only in antigens of the second type, *e.g.*, in iodo-BSA-I[131], is the radioactive trace element present in the determinant haptenic group of the antigen molecule.

Elimination of the antigen from the blood.—If rabbits are injected with trace-labeled serum albumin or serum globulin of other mammals, the injected protein remains in the blood in high concentrations for from three to seven days; it behaves, in this respect, similar to rabbit serum protein injected into rabbits (25, 26, 28, 32). The percentage of injected beef serum albumin found in the blood of rabbits on the first through the eighth day was approximately 50, 40, 35, 30, 27, 25, 19 and 13 per cent respectively (25). The sudden drop in the antigen level of the blood on the seventh day is caused by the formation of antibodies and by the removal of antigen-antibody complexes from the blood. In contrast to the serum proteins, ovalbumin from hens' eggs, when injected into rabbits, is rapidly removed from the blood; most of the radioactivity is found in the urine (32, 33). The same is true for the heavily substituted iodo- and azoproteins (29, 34, 35), and for the streptoccal polysaccharide A (36). The radioactivity of the urine is due to the excretion of split products of the antigen such as iodinated peptides (34). While the half-life of human serum albumin in the blood of rabbits is about five days, that of heavily substituted azoproteins is only 1.5 days (37).

Deposition of the antigen in organs and tissues.—If deeply colored azoproteins are used as antigens, their deposition in the macrophages of liver, spleen, bone marrow, and other cells of the reticulo-endothelial system is visible in histological preparations (38, 39). Intravenously injected azoproteins are found in the cardiac valves, in connective tissue, and in the lining of the hepatic sinusoids, whereas lymphocytes and lymphoblasts are free of antigen (38). Observation of the antigen in the ear-chamber preparation of rabbits reveals that shed fragments of the cells, which contain the colored antigen, are ingested by adjacent macrophages (39). This is a nonspecific reaction as shown by the analogous phagocytosis of injected bacilli in normal rabbits (40). Since the small hapten molecules, in contrast to the large antibody molecules, are neither deposited nor able to elicit antibody formation (29, 38), deposition of the antigen seems to be one of the prerequisites for its antigenic action.

The organ and tissue distribution of colorless antigens can be determined by tagging with isotopes and measuring the radioactivity of the tissue proteins; radioactive metabolic break-down products have to be removed by extraction with trichloroacetic acid and organic solvents (27, 29, 32, 35). The same method can be applied to fractions obtained from tissue homogenates by fractional centrifugation. It was found in this manner that intravenously injected heavily substituted iodoproteins are first deposited in the microsomal fraction, *i.e.*, in the submicroscopic cytoplasmic granules of rabbit liver and spleen (35). About 20 to 40 minutes after injection the highest concentration of antigen is found in the mitochondrial fraction which con-

tains the large cytoplasmic granules, while the antigen concentration in the proteins of the nuclear fraction and in the soluble proteins of the cell sap is low (35). Similarly, anthranil-azo-ovalbumin-C^{14} (29), and sulfanilazo-serum globulin-S^{35} (27), are concentrated in the cytoplasmic granules.

In contrast to the heavily substituted protein antigens, which disappear rapidly from the blood, the deposition of the lightly substituted antigens is obscured by the high antigen content of the blood. Even if blood and lymph are removed by perfusion of the tissues with isotonic saline solution or by washing of the homogenates, antigen is found in almost equal distribution in all of the subcellular fractions; after several weeks, however, when the antigen level of the body fluids is low, most of the deposited antigen is again found in the mitochondrial fraction of the antigen-containing tissues (27, 41, 42). This fraction, obtained by high-speed centrifugation, consists mainly of cytoplasmic granules of approximately 1 μ in diameter. If the organism is flooded by very large amounts of protein or azoprotein (*e.g.*, 20 mg. per mouse), some of it is found in granules which are not stained by mitochondrial stains (43, 44). Streptococcal polysaccharide A (36), and herpes virus (45) behave in the same manner as the soluble protein antigens, being deposited in the cytoplasm, particularly in the mitochondria. Coons and his co-workers (46) have found antigen not only in the cytoplasm, but particularly in the nuclei of macrophages, monocytes, and reticular cells; the antigen was made visible by treating frozen sections of the organs with fluorescent antibody, with observation of fluorescence under the microscope (46a). The high concentration of antigen found in the nuclei by means of this method does not agree with the higher cytoplasmic concentration found by other methods (39, 44).

If rabbits are injected with nonradioactive iodo-ovalbumin, and reinjected 3 to 4 days after the last injection of the antigen with radioactive iodo-ovalbumin-I^{131}, the distribution of radioactivity in organs and subcellular fractions is the same as in untreated rabbits (35). Injection of human serum albumin-I^{131} into rabbits or mice 10 days after the last injection of nonradioactive serum albumin causes increased deposition of the antigen in the mitochondrial fraction of the tissues (41).

Persistence of the antigen.—It is of the utmost importance for our understanding of the immunological response to know whether or not the presence of antigen is necessary for antibody formation. Antibodies against the pneumococcal polysaccharides II and III have been found in human beings 8 years after sensitization (47). Likewise, it is well known that immunity against virus infections lasts many years and that antibody production goes on for all this long period of time. Indeed, *Rickettsia orientalis* has been demonstrated in the blood and tissues of infected mice up to 610 days after injection (48). While this may be due to multiplication of the virus *in vivo*, such an explanation cannot be applied to inanimate antigens such as polysaccharides or proteins. Pneumococcal polysaccharide injected into mice was

found six months after injection by means of fluorescent antibody (46). Likewise beef serum gamma globulin or azoproteins injected into donor mice were found in the organism 85 to 120 days after injection, use being made of the vascular ear reaction in sensitized recipient mice (49). Trace-labeled anthranilazo-ovalbumin was found in rabbits five months after injection (29, 50), sulfanil-azo-globulin in mice 6 to 7 months after injection (27).

Experiments with trace-labeled antigens permit us to measure the rate of disappearance of antigen from the sites of deposition. Such experiments reveal that an initial period of rapid disappearance of the antigen from the tissues is followed by a very slow rate of elimination (27, 29). The initial rapid elimination of antigen apparently results from the fact that the organism, immediately after injection, is flooded by an excess of antigen. Only a small portion of this antigen is absorbed or deposited while the bulk remains free and is rapidly eliminated from the body. A rough calculation shows that the average antigen content in rabbits injected with 30 mg. per kg. iodo-ovalbumin-I^{131} is approximately 10^6 antigen molecules per liver cell; one month after injection, when the antigen content is reduced to about 10,000 molecules per cell (51), the rate of elimination is considerably decreased (27, 29). Evidently, only the latter very small amount of antigen is significant for the process of antibody formation. In order to avoid the unnatural first phase of enormous antigen excess, the amount of injected antigen can be reduced from the usual 20 to 40 mg. per kg. animal to about one thousandth of this amount. If rabbits are intravenously injected with such small amounts of iodo-ovalbumin-I^{131}, a considerable portion of the antigen deposited in the liver is again found in the cytoplasmic granules (52). Reports on the absence of antigen in liver and spleen a few weeks after injection of 15 to 60 mg. per kg. are due to insufficient sensitivity of the analytical methods.

During the first few days after sensitization, when large amounts of antigen are present in the blood and antibody is being released into the blood, insoluble antigen-antibody complexes may be formed and deposited in the reticulo-endothelial system (53). The rapid elimination of such complexes from the blood is not surprising since other colloids such as silver chloride or gold are also rapidly eliminated, their half-life in the blood being approximately 50 sec. (54).

While circulating antibody can thus remove the circulating antigen from the blood, it seems that intracellular antigen is inaccessible to circulating antibody (55). Only extracellular or adsorbed T_2 phage seems to react with the homologous antibody (56). Nevertheless, the deposited antigen is able to elicit antibody formation when it is released from the cells. This has been demonstrated by sensitizing rabbits with diphtheria toxoid and injecting cells of their lymph glands or spleen into other rabbits; although the injected cells had been washed in order to remove soluble antigen, antibody production in the second animal was elicited and reached a peak about four weeks after injection (57).

ANTIBODIES

Site of formation.—The site of antibody formation depends on the locus of entry of the antigen. When *Shigella* was injected into the foot pad of rabbits, antibodies were first found in the popliteal lymph nodes (58). Similarly, secondary stimulation by the subcutaneous injection of tetanus toxoid with alum to horses, rabbits, and guinea-pigs led to the production of antitoxin in the lymph glands which drain the injected area (59). Antibodies to equine encephalomyelitis virus, a neurotropic virus infecting mice, are produced in the brain (60). The local production of antibodies is proved impressively by the finding of anti-ovalbumin in the right, and of anti-human serum albumin in the left cornea of a rabbit, 192 hr. after intracorneal injections of the corresponding antigens (61).

The role of the spleen in antibody production varies during the immunization process. Initially, most of the antibody in rabbits is formed in the spleen (62) so that the antibody titer is reduced when splenectomized animals are given a single injection of the antigen (63); later, an "inactive phase" follows in which the spleen stops forming antibodies and antibody production is taken over by other organs (62). If the antigen is given intraperitoneally splenectomy does not affect the antibody titer in rats (64). In chickens, antibody production is impaired by splenectomy (65).

The wealth of lymphoid cells in the spleen and the finding of antibody in regional lymph nodes suggested that antibody production might take place in lymphocytes (66). However, quantitative investigations show that the antibody content of lymph and lymphocytes is never higher than that of the blood (67, 68), so that formation in the lymphocytes is improbable (69). Since the formation of antibodies coincides with the appearance of plasma cells, it has been concluded that the plasma cells are the site of antibody production (70, 71, 72). Investigations of Fagraeus (71) of rabbits injected with horse serum show that reticulum cells undergo conversion into "transition cells" which stain red with methyl green-pyronine, and blue with the Giemsa stain; the transition cells are converted into immature and later into mature plasma cells (71). After intravenous injection of the antigen, plasma cells are found not only in the spleen, but also in the liver, the lung, and the bone marrow (73). Local administration of the antigen leads to hyperplasia of the neutrophil cells and to increased production of lymphoblasts, plasma cells, large lymphocytes, and reticulum cells in the regional lymph nodes, changes which are specific for antigen action, and cannot be called forth by nonspecific agents such as turpentine (74, 75). Investigation of the splenic plasma cells with the phase microscope reveals the formation of large drop-like formations in their cytoplasm. The nature of these drops is not clear; they cannot be stained by the common stains (76). It is remarkable that granules similar to those found in plasma cells are also found in myeloma cells which are responsible for the excessive formation of globulins in myeloma patients; this suggests that the granules are involved in the production of globulins and of antibodies (76). Extraction of subcellular fractions of

tissue homogenates with 0.9 per cent saline solution indicates the presence of antibody in the mitochondrial fraction, 48 hr. after injection of the antigen (77). No antibodies were found in the hemolymph of caterpillars injected with various antigens (78).

Rate of formation and break-down of antibodies.—Previous experiments with N^{15}-labeled amino acids had proved that these amino acids are incorporated into antibodies at approximately the same rate as into normal serum globulins, but that they do not become part of preformed antibodies in passively sensitized animals (79). This is confirmed by the administration of carbon-labeled lysine (80), leucine (81), valine (82), and glycine (83) to rabbits sensitized by the simultaneous injection of pneumococcal polysaccharides (80, 82), ovalbumin (83) or arsanil-azo-ovalbumin (81). The experiments performed with C^{14}-lysine are particularly valuable since this amino acid cannot be formed *in vivo* by transamination of the corresponding α-keto acid. It was found that the ratio between radioactivity and the antibody titer remained constant when the serum of a sensitized rabbit was transfused to another normal animal. This proves that there is no exchange between the incorporated lysine molecules and the lysine pool of the body (80) and indicates that antibody molecules, and probably also other serum globulin molecules, are not rearranged *in vivo* or rebuilt by "hopping-in-and-out" of amino acids, but are formed within a very short period of time and are also completely broken down within a very short time. In man, the average half-life of an antibody molecule is approximately 15 days; in beef, 21; in dogs, 8; in rabbits, 5; in guinea pigs, 4.5; and in mice, 2 days (84). The half-life of antibodies in newborn children is 20 to 30 days (84, 85). In rabbits, the half-life of antibodies is longer than that of other serum globulins, but shorter than that of albumins (82).

If spleen or liver slices of rabbits sensitized by human serum globulin are incubated with C^{14}-glycine, the radioactive amino acid is incorporated into the antibodies formed (86, 87). The antibody production per mg. of spleen tissue is far superior to that of liver tissue (87). *In vivo*, however, the liver may have the same importance as the spleen since its weight in rabbits is approximately 30 to 50 times higher than that of the spleen. While incubation experiments indicate the formation of antibody directly from amino acids and not by the rearrangement of other globulin molecules, *in vivo* experiments with administration of C^{14}-amino acids resulted in the formation of antibodies with C^{14}-content higher than that of the free amino acids (83). This would indicate formation of antibodies from other proteins rather than directly from the administered amino acids.

External factors affecting antibody production.—Formation of antibodies is reduced in animals suffering from deficiency of folic acid, riboflavin (88) or vitamin B_6 (89), and in malnourished human beings (90). On the other hand, diphtheria antitoxin is formed even in nutritionally depleted persons up to their death (91). The ability to form serum globulins is not paralleled by the capacity to produce antibodies; myeloma patients with high serum globulin

content were poor antibody producers, whereas normal antibody response was found in cirrhotic patients (92).

In guinea pigs antibody production was inhibited by the horizontal bisection of the cervical medulla; hence, the central nervous system seems to be necessary for sensitization (93). The antibody formation seems also to depend on the external temperature; among mice injected tetanal toxoid at 5°, 25°, and 35°, respectively, the last group had the highest antibody titer (94). Frogs produce agglutinins at 20° but not at 8°C.; by warming up the animals to 20°, the antibody titer is restored (95).

While some authors report an enhanced antibody production by the spleen upon administration of adrenal cortex extracts, and a reduced antibody production in the spleen of adrenalectomized animals (96), most investigators find a reduction in the antibody titer on administration of cortisone (97, 98, 99). This is attributed to a decrease in the plasma cell reaction (100). Similar effects, resulting in lower antibody response, are brought about by the pituitary adreno-corticotropic hormone (ACTH) (97, 100 to 104). Cortisone inhibits not only primary antibody production but also the secondary response (105); it does not affect the rate of disappearance of antibodies (105), and does not seem to have any influence on the combination of antigen with antibody *in vivo* (106). Previous reports on an anamnestic rise in the antibody titer occasioned by ACTH could not be confirmed (107).

The action of irradiation with x-rays on antibody production depends on the time of irradiation. If rabbits are exposed to x-rays in a period beginning two days before and ending 2 hr. after injection of the antigen, antibody formation is considerably reduced (108, 109). This adverse effect of irradiation can be prevented by shielding the spleen (110), even if the spleen is removed 24 hr. after irradiation. It is concluded that the spleen releases substances required for antibody production in other organs (110). Irradiation more than 2 hr. after injection of the antigen does not seriously affect antibody production (108, 109). Neither phagocytosis of gold particles nor the anamnestic response are much affected by irradiation with x-rays (108). Moreover, antibody production goes on after destruction of the lymphoid tissue by x-rays. Evidently, only the first phase of antibody production is sensitive to the action of x-rays. It has been suggested that this phase consists of the formation of a modified "γ-globulin generator" (108).

If mice are injected with large amounts of pneumococcal polysaccharide, a state called "immunological paralysis" results (111). During this paralysis, which lasts several months, the animals do not respond to immunizing doses of pneumococcal polysaccharide. It seems that the immunological paralysis is due to exhaustion of the reticulo-endothelial system by excessive doses of the antigen. Indeed, large amounts of the polysaccharide have been found in the tissues during the period of immunological paralysis. The inability of the organism to form auto-antibodies has been attributed to a similar mechanism, viz., to the saturation of the antibody producing apparatus by body constituents (112). The suggestion has been made that erythroblastosis in

newborn children might be prevented by the administration of large amounts of blood group antigen (113).

Labeling of antibodies and quantitative determination.—While antibodies in the body fluids of passively sensitized animals can be determined by the customary routine methods, it is difficult and frequently impossible to determine antibodies which are adsorbed to the tissue and cannot be extracted. This difficulty can be overcome by using isotopically labeled antibodies. If only traces of the radioactive isotope are coupled to the antibody molecule, affinity for the antigen is practically unchanged, and the antibody content of tissues or precipitates can be determined by measuring their radioactivity. For such studies horse antibody against pneumococcal polysaccharide (114), diphtheria antitoxin (114), rabbit anti-human serum albumin (115), and anti-ovalbumin (116) have been labeled by coupling with traces of radioactive iodine I^{131}. Radio-iodine also has been used as a label for rabbit antibodies against azoproteins; in order to prevent iodination of the combining sites of the antibody molecules, an excess of an azo dye bearing the same hapten group as the antigen was added before iodination (117). Since the short half-life of radio-iodine is not favorable for radioautography, coupling of antibodies with traces of diazotized radioactive sulfanilic acid was used in such experiments (118). Antibody labeled by coupling with fluorescein isocyanate was used as a reagent for the localization of antigen in tissue sections (119). The distribution of agglutinins in sections was also examined by treating the sections with an excess of the homologous bacteria and observing their agglutination on the slide (120).

Rabbit antiserum-I^{131} against human serum albumin injected into guinea pigs was eliminated from the blood after four days owing to the formation of antibodies against the rabbit protein (115). Antiserum-I^{131} against rat kidney, injected into rats, was deposited in the kidney, with smaller amounts in the lung and liver (121). The antibody could be split off from the kidney by perfusion with dilute alkali, and could in this fashion be purified from other proteins present in the anti-kidney serum (121). Antibodies against liver and lung were prepared in the same manner; the deposition of all these organ-specific antibodies occurs in the vascular bed of their target organs (121). The homologous antigens are heat-stable substances which can be extracted from the organs by alkali, but not by saline solution (122).

Chemical and physical-chemical properties.—The literature on the chemistry of antibodies has been reviewed by Smith (123), their physical chemistry by Williams (124). Antibodies can be purified either by nonspecific physical-chemical methods, or by specific combination with the homologous antigen and dissociation of the antigen-antibody complex. In the nonspecific methods, antibody is separated from other proteins of the immune serum by fractionation with neutral salts or ethanol, or by electrophoresis; fractionation of Rh sera by the new method of electrophoresis convection revealed that the Rh antibodies are distributed throughout several fractions of the serum globulins (125). The blocking antibodies were found in the γ-globulin frac-

tion, the cryptagglutinoids in the α-, β-, and γ-globulins (125). While most of the antibodies of horse sera are present in the euglobulins, which are insoluble at low ionic strength, the antitoxins were found in the pseudo-globulin fraction (126). In the sera of horses injected with rabbit serum globulin, precipitins of various reactivity could be obtained by separation of the euglobulins from the pseudo-globulins, but not by fractionation with ethanol (127). Neutralizing antibodies against Newcastle virus were found mainly in the γ_1-globulins of beef serum, whereas hemagglutination inhibiting antibodies were present in the γ_2-globulin fraction (128). Slight alkalinity up to pH 11.4 does not seem to inactivate antibodies (129).

Purification of precipitating antibodies from other proteins of immune serum is achieved by precipitation with the homologous antigen and dissociation of the precipitate. Dissociation of azoprotein-antibody complexes is accomplished by acidification with hydrochloric acid at low temperature (130); pure antibody is obtained in good yield. Anti-ovalbumin and anti-serum globulin were purified by the same method using ovalbumin or serum globulin coupled with diazotized arylamino acids for precipitation; the yield of purified antibody was small (131).

Earlier analyses (123) did not reveal any significant differences between antibodies and normal serum globulins. Likewise, the amino acid composition of normal γ-globulins from rabbit serum was similar to that of purified antibodies against anthranil-azo-beef serum globulin or p-trimethylamino-phenyl-azo-beef serum globulin (132). These analyses do not reveal anything about the sequence of amino acids in the antibody molecules. Determinations of the N-terminal amino acid, and the adjacent three amino acids, in normal rabbit serum γ-globulin and in anti-ovalbumin from rabbit-immune serum gave in both cases the sequence alanyl-leucyl-valyl-aspartic and possibly glutamic acid (133). On the other hand, more carbohydrate and lipid was found in the pseudoglobulins of dogs sensitized by pneumococcal polysaccharide type I than in those of normal dog serum (134).

While the purification of antibodies is desirable for many reasons, one must be aware of the fact that the methods used for purification may alter the activity and specificity of antibodies. Crystalline antibody to pneumococcal polysaccharide type I was found to be heterogenous, and to react differently in solubility and in protection tests (135). Changes in the reactivity of antibodies are also brought about by delipidation. Rabbit precipitins lose their precipitating action on ovalbumin and human serum albumin after delipidation at low temperature (136); the precipitation of human serum globulin by the homologous antibodies is not abolished but delayed (136). Similarly, agglutinins for staphylococci and pneumococci lose their agglutinating power after delipidation, although they still combine with the homologous bacterial antigens (137). The agglutinating power can be restored by adding lecithin or cephalin (137). On the other hand, extraction of the lipids does not impair the precipitating action of horse serum precipitins (138). Evidently the absence of lipids does not affect the combination of

antibody with antigen, but prevents in some cases the second phase of the antigen-antibody reaction by increasing the solubility of the antibody molecules, by causing aggregation of the serum proteins, and possibly also by distorting the structure of the native antibody molecules (136). Changes of a similar type can be brought about by cautious heat treatment of immune sera. Such inactivated sera can be reactivated by the action of trypsin; the enzyme causes disaggregation of large aggregates formed during the heat treatment (139).

Differences in reactivity.—The reactivity of antibodies formed in response to the injection of soluble antigens depends on the animal type, on the antigen used, on the site, mode and time of injection, and on many other factors. Most of the injected antigens have a variety of determinant groups A, B, C, etc., so that multiple antibodies of the types anti-A, anti-B, anti-C, etc. are formed even after the injection of a uniform antigen. Furthermore, antibodies of the same specificity, such as anti-A, may differ from each other by their more or less perfect adaptation to the determinant group A of the homologous antigen.

The importance of the site of injection is illustrated by the formation of precipitins after intravenous injection of ovalbumin to horses, but of antibodies of the antitoxin type after subcutaneous injection (140). Antitoxins are obtained in both cases when diphtheria toxoid is used as antigen (141), but the response is meagre following intravenous injection.

The type of antibody formed is changed when the antigen is injected with an adjuvant such as alumina cream. While precipitins are formed in horses after the intramuscular injection of human serum albumin, nonprecipitating antibodies are formed after the injection of the alum precipitated protein (142). Similarly, skin-sensitizing antibodies are formed in rabbits injected with alum precipitated ovalbumin, whereas no such antibodies are produced after the injection of alum-free ovalbumin (143).

The specificity of the formed antibodies is reduced on prolonged injection of antigen, as shown by repeated injections of hemocyanin (144). Wider reaction zones were also found after the prolonged injection of ovalbumin and conalbumin (145). Many of these effects are due to the multiplicity of antibodies and to the overlapping of their respective equivalent zones (146). Excess of one antigen in the equivalence zone of another related antigen may simulate the presence of nonprecipitating antibodies (146). Another reason for the decrease in titer and specificity on long lasting immunization is the presence of contaminants of high antigenicity. Frequently, antibodies of various reactivity are formed at different times; when influenza virus is injected into monkeys, hemagglutinating, complement fixing, and virus neutralizing antibodies are developed at different rates (147).

Of particular interest is the formation of nonprecipitating antibodies, called reagins, which are found in the α- and β-globulin fractions of the sera of allergic persons (148). As mentioned above (143), such skin-sensitizing reagins are produced upon administration of ovalbumin (148). Their forma-

tion after injection of diphtheria toxoid has been investigated with quantitative methods (149). It was found in these experiments that three types of antibodies are formed: (*a*) skin-sensitizing and precipitating antitoxins, (*b*) an intermediary type, and (*c*) nonprecipitating antitoxins. The first type contained 16 μg. of precipitable protein per antitoxin unit; the second type, 2 to 8 μg. protein. Only the precipitating antibodies were able to fix complement. The skin-sensitizing antibodies lost their sensitizing property and were converted into blocking antibodies by heating to 56°; their antitoxic and anaphylactogenic properties were not affected by heating (150).

The reagins can be differentiated from the precipitins, quite generally, by their lability against heat treatment (151). Contrary to the heat-stable antibodies, which after inoculation disappear rapidly from the skin, the reagins remain in the skin over long periods of time, causing skin sensitivity and allergic phenomena (151). Many other signs of clinical immunity or sensitization are due to the presence of reagins rather than to that of precipitins (151).

Nonprecipitating antibodies can be produced from precipitins by proteolytic digestion, *e.g.*, by the action of cathepsin on rabbit antibodies against ovalbumin (152). The digestion of diphtheria antitoxin by pepsin at pH 3.8 is not due to the action of pepsin but to that of cathepsin present in pepsin preparations (153). If immune sera against *Hemophilus pertussis* are digested by pepsin, the complement fixing antibodies are destroyed, but not the antitoxins (154).

Neutralizing, heat-stable antibodies, but not reagins, pass from mother to fetus (151). Surprisingly, human split antitoxins are not able to pass the placenta, whereas antitoxins are transferred from mother to child (155). In kids, colostral antibodies are mainly transported by the lymph, not by the blood; in rabbits, antibodies pass through the yolk-sac splanchnopleur (156).

Some confusion is caused by names such as univalent or incomplete antibody for the reagins. The inadequacy of these designations is evident from the peculiar properties of avian antibodies; in contrast to mammalian antibodies, the antibodies of chickens and other birds form antigen-antibody complexes which are less soluble at salt concentrations of 8 to 10 per cent than at 1.0 per cent (157, 158). The antigen-antibody complexes of mammalian antibodies display just the opposite behavior, being insoluble in isotonic saline but more soluble at high salt content. We have no reason to assume an increase in valency at high salt content in one case, a decrease in the other. It is much more probable that the solubility of antigen-antibody complexes depends on their physical-chemical properties such as the presence of hydrophilic or hydrophobic, lipophilic or lipophobic groups upon the surface of the antigen-antibody aggregates, and on the formation of salt bridges between anionic and cationic groups of adjacent antigen-antibody complexes.

Theories of antibody formation.—Since antibodies have all the properties of serum globulins, antibody formation is simply the formation of modified globulins. In contrast to views held previously, antibody formation cannot

be considered as a defense mechanism; indeed, antibody formation in many cases has harmful consequences for the organism (159). The specific combination of antibody with the antigen is generally ascribed to the presence in the antibody molecule of a group the shape of which is complementary to the determinant group of the antigen. Various hypotheses have been advanced in order to explain this structural adaptation of the antibody globulins.

According to the "template theory," the antigen acts directly as a mold, the antibody being formed as a negative print of the determinant group of the antigen (3) by a coin-and-die mechanism (160). Since the amino acid content, probably also the amino acid sequence, is the same or very similar in antibodies and normal serum globulins, it is assumed that the antigen does not interfere in the first phase of protein synthesis in which the species-specific peptide chain is formed, but that it interferes with a second phase of protein synthesis in which the peptide chain folds up to form the globular antibody molecule (3, 161). In view of the cytoplasmic deposition of antigen, it is probable that the action of the antigen takes place in the cytoplasm; the nonparticipation of the nucleus is in agreement with the fact that acquired immunity is never transmitted to the next generation (161). Various mechanisms for antigen action have been proposed, such as adsorption of the antigen to a monomolecular primary protein film (162), blocking of a pleated sheet template on which the antibody molecule is formed (163), organizer action of the antigen (164), and indirect template action by means of alternate formation of positive and negative replicas (165). Template action *in vitro* has been demonstrated in methyl orange and other dyes adsorbed to silica gel; if the dye is eluted from the gel, the gel seems to maintain an "anti-dye" structure which enables it to combine specifically with the previously adsorbed dye (166).

The main objection raised against the template theory stems from the long persistence of immunity, and from the belief that antigen cannot persist in the organism over the whole period of immunity (167). In order to explain the formation of antibodies after disappearance of antigen, it is assumed that the antigen modifies the "γ-globulin generators" (108), *i.e.*, the globulin producing enzymes, so that these produce antibodies instead of normal globulins (167). The role of the antigen, according to this view, is similar to that of the substrate in the process of enzyme adaptation; since it is well known that enzyme adaptation is reversible in the absence of substrate, the additional assumption has to be made that the adapted, antibody producing enzyme remains adapted after elimination of the antigen. Attempts were made to prove the presence of such antibody generators by the inoculation of antibody producing tissues from immunized to normal animals (168). However, only in those instances where living antigen had been used was antibody production discovered in the second animal (169). Evidently, virulent microbial antigens had survived and were transferred with the inoculated material, giving rise to active immunization (169).

In view of the persistence of latent virus in mammals (170) and of the

p'ersistence of injected protein and carbohydrate antigens in the reticulo-endothelial organs of injected animals (27, 29, 35, 46, 49), it is very doubt-ful, however, that antibody production continues after elimination of all of the antigen (171).

ANTIGEN-ANTIBODY INTERACTION

Combination of antibody with soluble antigens (171a).—The combination of precipitins with soluble antigens depends on the salt content of the medium (172), the temperature (173, 174), pH (175), on the antibody/anti-gen ratio in the solution and on other physico-chemical conditions. Thus, antibodies from frog serum react only at temperatures above 10°C. (174), and chicken antibodies give precipitates best in solutions containing 8 to 10 per cent NaCl (172). Crystalline carboxypeptidase combines at room or body temperature with one, at 6.4°C. with two antibody molecules (173).

Inhibition experiments with various haptens show that the combination of antibody with antigen is brought about mainly by van der Waals forces (176), which become important when the closest fit occurs, *i.e.*, over short distances (177). The mutual attraction of antigen and antibody through plastic screens over distances of about 100 Å (178) is attributed to inhomo-geneity of the films (179) and to penetration of the antigen through the film if its thickness is less than the "critical thickness" of about 150 Å (180). If horse antibody combines with monomolecular films of azo-ovalbumin con-taining from zero to 60 determinant groups per antigen molecule, the thick-ness of the antibody layer is proportional to the number of determinant groups up to a maximum of 30 determinant groups where the thickness of the antibody layer is 270 Å. It is assumed that this is the length of the anti-body molecules which, at this point, are closely packed, their longitudinal axes being perpendicular to the surface (181).

If the combination of antibody with antigen occurs in solutions, the first phase of combination is followed by aggregation of the primary complexes and the formation of insoluble aggregates. Attempts have been made to measure the size of antigen-antibody complexes in the region of antigen ex-cess, where they are soluble, and to determine their antibody/antigen ratio. Indirect analyses after electrophoresis (182 to 184) and sedimentation in the ultracentrifuge (183, 185) show that the ratio of antibody/antigen in the soluble complexes varies from 1.0, at slight antigen excess, to approxi-mately 2.0 at high antigen excess; similar results are obtained by evaluation of old experiments (186). Hence, the composition of the soluble complex is represented by formulas varying from G_nB_n to $G_{2n}B_n$, where G=antigen and B=antibody molecule. While these experiments do not give any informa-tion on the magnitude of n, ultracentrifugal determination of the size of the complex rabbit antibody-beef serum albumin indicates that n is very small; the formula of the soluble complex is G_2B or G_4B_2 (183).

Since antibody, even at very large antigen excess, combines with only two, in some cases three or four antigen molecules, the valence of antibodies,

i.e., the number of specifically combining groups per antibody molecule, cannot be high, and is, most probably, one or two. In the insoluble precipitates, on the other hand, the antibody/antigen ratio is very high, corresponding to formulas varying from G_nB_{2n} to G_nB_{80n}. Formulas with high B/G ratios suggest that antibodies are univalent and that the precipitate consists of aggregates, each of them containing one antigen molecule to which many univalent antibody molecules are bound. The aggregates are probably held together by nonspecific forces. Most investigators attribute the formation of precipitates to the action of bivalent antibody molecules, *i.e.*, to specific forces (171a, 186, 187). This view is supported by the observation that the antigen-antibody aggregates maintain their large size even if they are dissolved by the action of acid, alkali, urea, or thiocyanate (184). Bivalence of antibodies is also supported by the observation that antibodies, elicited by injection of arsanil-azo-horse serum globulin, are able to combine at the same time with horse serum globulin and with arsanil-azo-hapten, and that antibodies against iodohorse serum globulin combine simultaneously with horse serum globulin and diiodo-tyrosine (188). It is hardly possible, however, to exclude in such experiments overlapping of the specific antigen-antibody reaction by nonspecific combination with further antigen or hapten molecules. Nonspecific adsorption of hapten to proteins is well known (189). Moreover, it seems that the forces operating between antigen-antibody aggregates are weaker than those responsible for the formation of the primary antigen-antibody complex (184).

If iodoprotein is precipitated by a large excess of anti-iodoprotein, the precipitate, the surface of which is formed by antibody molecules, does not combine with added diiodotyrosine-I^{131} (190); this supports the view that the precipitating antibodies are univalent. Attempts to prepare heteroligating antibodies, *i.e.*, antibodies containing two combining groups of different specificity, failed (191). It seems to the reviewer that all these experiments are reconcilable with univalence as well as with bivalence of the precipitating antibodies, and that we are not yet able to decide whether the formation of the precipitate is due to specific or nonspecific combination of the small primary antigen-antibody complexes.

Agglutination.—While the agglutination of red blood cells by agglutinins is a well-known phenomenon, the importance of nonagglutinating antibodies, particularly Rh antibodies (112, 192), has been discovered only recently. Although these antibodies combine quite specifically with red blood cells, they are not able to cause visible agglutination. According to the reactions used for their detection they have been called cryptagglutinoids, third-order agglutinins, agglutinoids, incomplete antibodies, glutinins or blocking antibodies (193, 194).

The mode of action of the agglutinins is not yet well understood. While the surface of normal red blood cells displays a smooth surface in electron micrographs, a thick coating layer indicating a drastic molecular rearrangement is visible in the agglutinated cells of sludged blood (195). Owing to the

large size of the red cells, structural details cannot be seen. Electron micrographs of the much smaller bacilli reveal, however, that the agglutinating antibodies combine with the carbohydrate structure of the bacillus (196). The same is probably valid for the agglutinins of red blood cells.

It has been frequently assumed that agglutination depends on the presence of bivalent antibodies which are able to combine simultaneously with two adjacent cells, whereas combination of cells with univalent antibodies is not followed by agglutination. This view is supported by the formation of separate agglutinates when a mixture of two types of cocci is treated with a mixture of the homologous agglutinins, while mixed agglutinates are formed when the same cocci are coupled with diazotized atoxyl and treated with anti-atoxyl serum (197). However, the type of agglutinate formed depends also on the concentration, higher concentrations favoring the formation of mixed agglutinates (198). Agglutinins in sera of the type O have been considered as bivalent because absorption of such sera with cells of types A or B and subsequent warming of the cells releases antibodies which are able to agglutinate cells of both types A and B (199).

In view of these and similar observations (200), it must be remembered that erythrocyte suspensions owe their stability to negatively charged groups in the surface of the cells (201) and that loss of these charges necessarily leads to agglutination. The loss of negative charges may be brought about by nonspecific combination with a positively charged colloid or by specific combination with an agglutinin.

The inadequacy of the designations univalent and bivalent antibody for nonagglutinating and agglutinating antibodies, respectively, is apparent from the fact that red blood cells, after exposure to the action of trypsin (202 to 204) or papain (205 to 207) are agglutinated by nonagglutinating antibodies. It is not quite clear whether the enzymes uncover further antigenic groups so that more antibody molecules can be bound (204), or whether they remove inhibiting substances from the surface of the red cells (207). The first of these two explanations is in better agreement with the fact that sensitized cells agglutinate when they are mixed with enzyme-treated normal cells (205).

Nonspecific agglutination of red blood cells of the chicken is also brought about by certain viruses (208). Here, too, agglutination of normal red cells of the fowl is observed when they are mixed with fowl cells which were exposed to influenza virus at 37° (209). The agglutinating action of influenza virus is inhibited by blood group substances A and O, by glandular mucin, but also by gum acacia and by pectin from fruits (210, 211). Apparently, these polysaccharidic acids compete with a similar substance of the red cells for the virus. The combination of the virus with red blood cells depends on the presence of ions, bivalent ions being more efficient than univalent ions (209, 212); the virus is split off by salt-free water (209). If the mucopolysaccharides of the red cells are oxidized by treatment with periodate, the virus is still bound but cannot be eluted (213).

Suspensions of red blood cells which are sensitized by combination with nonagglutinating antibodies, can be agglutinated in some cases by a heat-labile nonspecific factor of blood plasma (214, 215). This factor, called by Bordet "conglutinin," is related to complement but is not hemolytic. It seems that the fraction C'3 of complement is not involved in conglutination, but that the other fractions are bound in the order C'1, C'4, C'2, prior to the action of conglutinin (216). While the conglutinin of bovine blood plasma is inactivated at 56° (214), some of the sensitized red blood cells are agglutinated by heat-stable protein fractions of the blood serum (215, 217) or by colloids such as the sulfuric acid ester of polysaccharides (218, 219), gum acacia (198), or polyvinylpyrrolidone (218, 220). The conglutinating action of colloids depends on their molecular weight, with an optimum at a value of approximately 35,000 (220). The nonspecific conglutinating action of these colloids indicates that the red blood cells, after combination with non-agglutinating antibodies, are labilized, probably by the loss of some of their negative charges (112).

If foreign antigens are combined with red cells, agglutination of the red cells can be produced by precipitating antibodies against the foreign antigens. Thus, red cells treated with the carbohydrates of *H. influenzae* or *Shigella dysenteriae*, (221), or with the *Vi* or *O* antigens of *Salmonella typhosa* (222), are agglutinated by antibodies against these polysaccharides. If a protein antigen does not combine directly with red cells, combination can be accomplished by treating the red cells first with tannin and then with the protein; addition of the homologous anti-protein then causes agglutination (223). Traces of antibodies to arsanil-azo-ovalbumin were determined by the agglutination of red blood cells which had been sensitized by combination with arsanil-azo-protein (224). Bovine red cells, which are not agglutinable by antibodies formed in rabbits, are agglutinated by alternate treatment with globulins and anti-globulin serum (200). By means of this very sensitive method it is possible to detect nonagglutinating antibodies which cannot be detected by any of the other tests mentioned in the preceding paragraphs (enzyme test, conglutinin test, blocking action) (225, 225a).

Hemolysis.—The literature on complement has been reviewed recently (226). The mechanism of complement action, and the significance of its four fractions is still obscure. The complements from horse, cow, sheep, and guinea pig serum consist of the same four fractions; in contrast to guinea pig complement, however, the complements of horse, cow, and sheep do not hemolyze sheep red cells sensitized by the immune serum of rabbits (227). The conglutinin action of complement has been mentioned in the preceding section; it seems that this action is chiefly caused by the component C'4, but that it is counteracted by C'1 (228). If the serum is fractionated by the ethanol procedure, complement is found in fraction III-2, together with prothrombin (229). When prothrombin is converted to thrombin, complement disappears. On the other hand, prothrombin is still present when complement is bound by an antigen-antibody system (229).

If antibody combines in the living organism with antigen or hapten, complement is bound and disappears from the circulating blood; this disappearance is also observed on reinjection of the antigen (230). Regeneration of the complement takes place within a short time; 18 hr. after the intraperitoneal injection of ovalbumin to guinea pigs which had been injected intravenously with rabbit antiovalbumin, the complement titer of the blood had regained its normal level (231).

If the complicated system of cellular antigens (erythrocytes) is replaced by homogeneous, soluble antigens, it is found that each part of precipitated antibody is able to bind 10 to 13 parts of complement (232). However, the minimum ratio of complement/antibody required for the lysis of red blood cells is much higher. While only 50 antibody molecules per red cell are necessary, the minimum number of complement molecules is about 25,000 to 60,000 (233). The very low number of antibody molecules probably indicates the transfer of antibody from cell to cell, so that each antibody molecule can act repeatedly (234). It has been suggested that antibody acts as an enzyme, complement as a coenzyme (235).

It is remarkable that arsanil-azo-sheep erythrocytes, sensitized by arsanil-azo antibodies from rabbit blood, are lysed on addition of complement, although the antibodies combine with the arsanil-azo groups, not with the agglutinogens of the sheep red cells (236). Likewise, bactericidal action has been observed, when *Salmonella* coated with antigens from *Escherichia coli* was exposed to antibodies directed against *E. coli* (237). According to these results, combination of antibody with integral portions of red blood cells or of bacteria is not a prerequisite for lysis or bactericidal action; the essential process consists, apparently, in alterations of the cellular surface, caused by its combination with antibody.

In vivo reactions of antibody with antigen.—Anaphylactic phenomena are produced not only by precipitating and agglutinating, but also by nonprecipitating antibodies (238). Quantitative determinations of the antibodies in the sera of animals sensitized against ovalbumin (238) or bovine serum albumin (239) indicate that the intensity of the anaphylactic reaction depends upon the antibody titer of the blood. Anaphylactic symptoms have been produced in sensitized guinea pigs by the injection of purified precipitins (240). These experiments prove that the anaphylactic reaction is caused exclusively by the antibody molecules and that none of the other constituents of the immune serum is involved in the reaction.

If isotopically labeled beef serum γ-globulin is injected into sensitized rabbits, the antigen is eliminated more rapidly from the blood (241) and is also catabolized more rapidly than in normal rabbits (242). Injection of the same antigen to sensitized guinea pigs shows that the amount of antigen deposited in the liver is the same as in normal guinea pigs, but that more antigen is deposited in the lung of the sensitized animals (243). When diphtheria toxoid was injected into sensitized individuals, much more antibody was found in the blood serum than after the first injection (244). This "anam-

nestic" secondary response possibly results from an intracellular antigen-antibody reaction which, by disruption of subcellular structures, causes release of intracellular antibody into the blood serum. Tissue lesions caused by the *in vivo* reaction of antigen with antibody have been demonstrated in sensitized rabbits injected with serum albumin or serum globulin (245). Since the appearance of these lesions was accompanied by disappearance of complement from the tissues, it was concluded that complement was involved in the anaphylactic tissue lession (245).

The assumption that the anaphylactic symptoms are caused by the release of histamine is supported by the chromatographic demonstration of histamine in the tissues during anaphylactic shock (246). A role of histamine in the anaphylactic phenomenon is also indicated by the antagonistic action of antihistaminics. It is surprising, however, that a more powerful antagonistic action is exerted by vitamin B_{12}; 30 μg. of the vitamin are able to protect guinea pigs against lethal anaphylactic shock (247). Although the muscles are impermeable to the large molecules of most antigens, the smooth muscle of sensitized guinea pigs reacts almost instantaneously with antigen; possibly this is a result of an action of the antigen on nerve cells, the release of histamine being considered as a secondary reaction (248). In rats, rabbits, and dogs, anaphylactic shock is accompanied by release of heparin into the blood (249). However, there is no increase of plasma proteases during the anaphylactic shock (250). Extensive reviews on allergy have been published (251, 252).

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BIOLOGICAL NITROGEN FIXATION—
A REAPPRAISAL[1]

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Since the review by Virtanen (87) in the *Annual Review of Microbiology* closed with March, 1948 it might be expected that this review should cover, insofar as possible, publications in this field since that date. Lochhead's (55) article on *Soil Microbiology* in last year's *Annual Review of Microbiology*, however, discusses adequately most of this material. The reviewers decided, therefore, to bring up-to-date their initial collaborative summary published in the *Annual Review of Biochemistry* in 1945 (12). Except for a section at the end which can be regarded as an afterthought, we shall follow the pattern of the 1945 review with the aim of recording modifications and extensions in our views made possible and necessary by the added knowledge gained from the research of the past eight years.

This choice of material necessarily limits the literature to those publications related to the specific topics discussed and also will require some duplication of bibliographic items cited by Lochhead (55), although an effort has been made to keep these to a minimum. In addition to Lochhead's review (which is especially recommended for its discussion of the applied phases of nitrogen fixation) a few others should be noted. Allen & Allen (1) have provided a comprehensive review of the physiology of the root nodule bacteria; Thaysen (81) has surveyed the anaerobic nitrogen fixers; Wilson (98, 99) has treated the biochemical aspects of fixation by the common agents. The most recent (November, 1952) is that of Jensen (42), published in Danish. The material in Waksman's (92) long-awaited text on soil microbiology is chiefly of historical interest. Isotopic research and its implications for studies of the past have been completely ignored; it is not surprising, therefore, to find a listing of alleged nitrogen fixers that includes *Diplococcus pneumoniae* but not the photosynthetic bacteria. Neglect of the results obtained with this powerful modern tool for exploration of biological mechanisms gives a curious, almost archaic, quality to this portion of the discussion.

AGENTS OF FIXATION

Methods.—Demonstration of nitrogen fixation by biological agents always has been closely associated with questions of methodology (97, 98). The isotopic method introduced in the early forties furnishes a sensitive and reliable technique provided adequate positive and negative experimental and analytical controls are included (12, 98, 99). This method requires care that conditions of culture be chosen which will not interfere with expression of weak nitrogen-fixing ability in an organism (e.g., aerobic vs. anaerobic conditions). To overcome this necessity for establishing suitable cultural

[1] The survey of literature pertaining to this review was concluded December, 1952.

conditions in a closed system, Newton (60) developed a method based on isotopic dilution in which the agent to be tested can be grown in the manner it is normally cultured in the laboratory. The organism is labeled by growing it in a suitable medium containing $N^{15}H_4^+$. It if does not use NH_4^+ as a source of nitrogen, it seems highly unlikely that it will fix N_2. Of course this does not limit the application of the method, as an organism can be supplied labeled organic nitrogen compounds instead of $N^{15}H_4^+$. The concentration of excess N^{15} is estimated in an aliquot of culture, and the remainder of the labeled culture is then exposed to ordinary N_2 in an environment best suited to its growth, i.e. air, if an aerobe, tank N_2, if an anaerobe. After incubation, the N^{15} is again determined in the culture to learn if, through fixation of ordinary N_2, any dilution of the label has occurred. The method can readily detect uptake of as little as 0.1 to 0.2 μg. N/ml., a sensitivity about 0.1 that of the gasometric method employing N^{15} but still 10 times more sensitive than the conventional Kjeldahl method.

Another novel method for detection of nitrogen fixation based on use of the mass spectrometer has been described by Sisler & ZoBell (74). A hydrogen-utilizing species of *Desulfovibrio* was shown to use atmospheric nitrogen by incubating for 40 to 60 days in a N-free medium under an atmosphere containing H_2, CO_2, N_2, and A, and measuring the decrease in the N_2/A ratio.

FREE-LIVING MICROORGANISMS

Bacteria.—One of the major developments in biological nitrogen fixation during the past three years has been the discovery of numerous new species of nitrogen fixers. As many of these have been already referred to in Lochhead's review (55), we shall merely call attention to the fact that they are grouped primarily among photosynthetic organisms, both bacteria and blue-green algae. Ability to use molecular nitrogen has been verified for the following genera of the photosynthetic bacteria through use of the isotopic procedure: *Rhodospirillum*, *Chromatium*, *Rhodomicrobium*, *Rhodopseudomonas* and *Chlorobium*.[2]

In the years 1949 to 1951 more genera of microorganisms were shown to fix N_2 than had been established during the previous sixty-odd years following the initial discovery by Hellriegel and Wilfarth in 1886. Existence of a group of bacteria which, although reasonably well-known, was unsuspected of having the ability to fix nitrogen spurred efforts to find other overlooked members in the biological world. Claims for fixation have been made and denied for a variety of organisms [for example (71a, 97)], but, so far as we know, verification by the critical isotopic test has not been attempted except in a few isolated cases. Among the most persistent but unverified reports in the literature are those claiming nitrogen fixation by species of *Phoma* (a mycorrhizal fungus) and by *Aerobacter aerogenes*. Our inconclusive experiments with *Phoma* using both isotopic and total nitrogen trials have

[2] Originally the culture of green sulfur bacteria tested by us was thought to be a species of *Chlorobacterium* and was so called in our publications; later it was identified as a species of *Chlorobium* (50).

been already discussed (99). Lately, we have examined a number of strains of *A. aerogenes*, one of which appears to fix slight but significant quantities of N_2; the uptake is too small to be detected by the Kjeldahl method but is readily picked up by the gasometric isotopic technique.

Blue-green algae.—Considerable attention recently has been paid to the nitrogen-fixing ability of the blue-green algae; Lochhead (55) cited three papers concerning these agents. Since his review, Okuda & Yamaguchi (64) have tested many algae collected from paddy soils and found fixation by several cultures. To minimize the possibility that fixation was effected by agents other than algae, cultures were tested both in light and dark; fixation in the dark was negligible. Among 643 cultures of blue-green algae collected from rice fields by Watanabe (94), only 13 fixed N_2; these nitrogen fixers appeared to be rare in northern soils but relatively abundant in tropical and semitropical regions. Four species were recovered in pure culture so their nitrogen-fixing capacity could be tested critically.

Williams and Burris (95) reported that of eight pure cultures of blue-green algae recently isolated from fresh lake water, two were nitrogen fixers, *Nostoc sp.* and *Calothrix parietina*. Trials with *Gloeotrichia echinulata* were inconclusive, for the culture had not been proved to be free of bacteria; subsequently, it has been purified and has been shown to fix nitrogen. Fixation in the families Nostocaceae, Rivulariaceae, Stigonemataceae and Scytonemataceae (all are members of the suborder Heterocystineae) appears to be firmly established.

EXCISED NODULES

Early attempts to demonstrate the fixation of nitrogen by nodules detached from the plant were frequently so erratic and subject to such large errors in sampling and analysis that the gains reported were unreliable. Interest in the possibility of obtaining such fixation was revived when Virtanen & Laine reported that the addition of oxalacetic acid to excised nodules assured consistent fixation. Extensive trials in our laboratory as well as by Allison and his collaborators to verify this claim were uniformly unsuccessful. Wilson (97, 98) provides a detailed discussion together with complete references to this early research. Hope was held that the use of N^{15}, which provides a far more sensitive test and one which is virtually free from sampling error, would reveal fixation by excised nodules. However, after a large number of tests with N^{15} Tove *et al.* (84) could write in 1950, "Proof of fixation and, even more important, definition of the necessary experimental factors that will insure its occurrence are not as yet realized experimentally."

Nevertheless, two years later Aprison & Burris (4) reported conditions under which they had obtained consistent fixation of N_2^{15} by excised soybean nodules. Field-grown soybean plants were denodulated in the laboratory, and within 3 to 5 min. after their removal from the plants the nodules were placed under an atmosphere containing N_2^{15}. After exposure, the nodules were ground with acid, centrifuged, and only the supernatant was analyzed for N^{15}. Fixation within 30 min. could be demonstrated readily. The speed of the fixation and the fact that the rate decreased markedly with

time attest that the fixation of nitrogen was accomplished by the nodules rather than by contaminating organisms. The consistent demonstration of fixation by excised nodules was attributed to (a) the use of vigorous field-grown plants, (b) the rapid exposure to N^{15} after excision, and (c) the analysis of only the soluble portion of the nodules. The experimental approach to a number of problems of symbiotic nitrogen fixation now should be simplified by the use of excised nodules rather than the intact plant.

Aprison (5) and Magee (56) have continued the study of detached nodules and have shown that soybean nodules 5 to 6 mm. in diameter fix nitrogen three to four times as rapidly as nodules less than 4 mm. in diameter. Nutman (63) had shown clearly that small nodules on red clover plants were less effective in fixing nitrogen than large nodules. The variety of soybean used (5, 56) had a definite influence on the fixation by its excised nodules, but the age of the plants in the range of 6 to 10 weeks had little effect. Slicing nodules decreased their fixation rate about fourfold (fixation still was readily demonstrable), and crushed nodules or nodules frozen in liquid air retained no detectable capacity for nitrogen fixation. Addition of a large variety of substrates, including oxalacetic acid, reducing agents, cofactors, and plant extracts alone and in mixtures, to sliced nodules, regularly decreased their nitrogen fixation rather than enhancing it. The optimum temperature for fixation by soybean nodules was 24 to 25°C., and the optimum level of oxygen was 0.22 to 0.25 atm. Bond (7) has demonstrated that nitrogen fixation is decreased when the supply of oyxgen to soybeans in culture solutions is reduced.

Data reported by Bond and others [see (97)] indicate that highly active nodules on soybean plants fix nitrogen equivalent to their own total nitrogen content in about two days. Although the fractions analyzed with excised nodules are admittedly different, in the best experiments (5, 56) with excised nodules the initial rate of fixation was sufficient to double the soluble nitrogen of the nodules in about 62 hr. Apparently for a short time after excision the nodules can fix nitrogen at a rate approaching that on the plant, but this capacity decreases rapidly (4).

PHYSIOLOGY OF THE ORGANISMS

Rhizobium.—The extensive review by Allen & Allen (1) together with the 1952 summary of Lochhead (55) provide recent detailed discussions of the general physiology of the root nodule bacteria; only a few typical papers that are significant for nitrogen fixation itself will be considered here. The role of molybdenum in the nitrogen nutrition of plants and bacteria continues to hold interest as does another hardy perennial, the life cycle of species of *Rhizobium*. Meagher, Johnson & Stout (57) report that the seeds of garden peas and beans may contain sufficient molybdenum for a complete life cycle. Demonstration of the need for Mo (as in reduction of N_2 or nitrate) may require use of seeds specially grown to reduce the molybdenum content. Whether essential or not, there appears to be little doubt but that addition of molybdenum salts to certain Australian soils increases the yields of legume field crops such as clover (3).

Bisset (6) from a detailed study of the complete and "reduced" life cycles of many strains of *Rhizobium* from both wild and cultivated leguminous crops concluded that one stage yielded gram-positive bacilli which produced endospores. If confirmed, this finding has important implications for the taxonomy of the organisms; for example, Bisset believes *Rhizobium* is closely related to the plant parasitic *Bacillus polymyxa* group.

Blue-green algae.—The algae have attracted attention as a potential source of food, and certain of the blue-green algae are particularly attractive because they can fix N_2. However, these organisms grow much more slowly than *Chlorella*. Faster growing strains may be isolated, or a careful study of cultural conditions might reveal how their rate of growth can be increased substantially. Eyster (21), studying the nutrient requirements of *Nostoc muscorum*, has demonstrated that it requires boron for growth; earlier (20) he demonstrated the necessity for Mn and Mo, and his medium also includes trace amounts of Cu and Zn. Neither Fogg (27) nor Magee (56) could achieve an increased growth of blue-green algae by inclusion of chelating agents in the medium, but this possibility should be explored further.

Because of the role excreted compounds have played in studies of the mechanism of fixation by the other agents, recent investigations dealing with the composition of the cells and their excreted products are of interest. Magee (56) has accounted for about 94 per cent of the nitrogen from hydrolysates of *N. muscorum*. The amino acid composition is not unusual; arginine is abundant, and as was reported earlier (95), methionine is present only in low concentrations. Purines and pyrimidines contribute about 5 per cent of the total nitrogen. Watanabe (94) qualitatively analyzed by partition chromatography the amino acids which could be extracted from blue-green algae. No appreciable excretion of nitrogenous compounds occurred from species of *Tolypothrix, Anabaenopsis,* or *Nostoc,* but *Calothrix brevissima* excreted free aspartic acid, glutamic acid and alanine into the culture medium.

Fogg (27) studied the nitrogenous compounds excreted by *Anabaena cylindrica* and found that 28 per cent of the total nitrogen was extracellular in 5 days but only 15 per cent in 15 days. Later (40 days), as the nutrient medium became deficient, the extracellular nitrogen made up 22 per cent of the total. Only small amounts of free glutamic acid, alanine and amides were found in the medium, but after acid hydrolysis a number of amino acids could be detected; serine and threonine were most abundant. Apparently the excreted nitrogen is largely polypeptide, and Fogg does not consider the compounds to be specific products of nitrogen fixation. Magee (56) found that less than 1 per cent of the total nitrogen was excreted by *N. muscorum,* and only a trace of free amino acids was present. In contrast, a strain of *Nostoc* studied by Henriksson (36) excreted from 19 to 28 per cent of its total nitrogen. Undoubtedly the organisms vary widely in their excretion of nitrogen, but cultural conditions also must drastically influence the amount of nitrogen released from the cells.

Azotobacter.—The most noteworthy development in the physiological studies of *Azotobacter* has been the recognition that this organism has

hitherto unexploited possibilities as experimental material for studies in comparative biology. The relatively large size of many of its species and strains recommends it to the cytologist concerned with the significance of various types of granules within bacterial cells, and its high rate of respiration (Q_{O_2} of 1,000 to 2,000) suggests its possibilities for the enzyme chemist. From an examination of the morphological cycle and nuclear apparatus of the three common species of *Azotobacter*, Pochon, Tchan & Wang (68) concluded that the organism is an excellent choice for examination of both microbial physiology and morphology. Eisenstark and his collaborators (16, 17) recognized four distinct morphological cell types but could not confirm the existence of a heat resistant spore stage. They also noted that some of the granules within the cells of *Azotobacter agile* 4.4 took nuclear stains and probably represented nuclear bodies.

As some of these granules likewise stain with preparations alleged to be specific for mitochondria (100), it was of interest to determine if cell-free preparations could be made that would behave as do mitochondrial preparations from tissues of plant and animal origin. Stone & Wilson (78, 79) found that such preparations from *Azotobacter vinelandii* would completely oxidize acetate with or without the particles (supernatant material after centrifuging from 2,500 to 25,000 g). The oxidation apparently proceeded by a conventional tricarboxylic acid cycle, contrary to earlier views based on work with whole cells. Extending these studies and using improvements in methods for preparation, Lindstrom (53) prepared and examined the properties of a "soluble" α-ketoglutaric oxidase. More recently Repaske (unpublished observations) has made similar investigations of a "soluble" succinic acid dehydrogenase from both *A. vinelandii* and *A. agile*. These enzymes are soluble in the sense that they remain in the supernatant after spinning for 30 min. at 144,000 g although about 40 per cent of the activity still is associated with cell particles that come down at various stages in the centrifugation procedures. It is clear, then, that both of these enzymes and probably others[3] can be dissociated by treatment into fragments very much smaller than the mitochondria of plant and animal cells.

Perhaps of more direct significance for nitrogen fixation as well as comparative biochemistry are the studies on the properties and functioning of hydrogenase in *A. vinelandii*. Hyndman *et al.* (37) report an iron containing component in cell-free preparations whose absorption peak shifted in the presence of H_2. Interpretation of this observation is difficult, since clearly the shifting peak could represent a carrier or a nonspecific reduction of some iron containing compound in the preparation as readily as the suggested prosthetic group of hydrogenase. Using modern physiological methods, including mutants, cell-free preparations, and the like, Green and co-workers (33) studied the occurrence of hydrogenase in *A. vinelandii* and confirmed

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[3] For example, Repaske (unpublished observations) has made a "soluble" preparation from *Azotobacter agile* by sonic disintegration followed by centrifugation at 144,000 g which will oxidize typical members of the TCA cycle including acetate when "sparked."

that the previously noted association of hydrogenase and nitrogenase was not an experimental artifact arising from faulty methodology.

Through use of deuterium gas, Pethica *et al.* (67) have confirmed that the action of hydrogenase in *Azobacter* is reversible; results to date, however, indicate that the rate of the exchange reaction is surprisingly slow in comparison with that leading to reduction of dyes, ferricyanide or molecular oxygen (37).

In this era of cell-free preparations, physiological mutants, isotopic tracing, and the like, it is refreshing that some workers recognize that a great deal of fundamental work remains undone for which less pretentious (and expensive) techniques are suitable. Even so "well established" a property of the *Azotobacter* as utilization of nitrate recently has been demonstrated not to hold for all strains (35). The study by Jensen (41) published under the old-fashioned but respected title of "Notes on the Biology of *Azotobacter*" is a welcome example of what can be done with a minimum of equipment.

Photosynthetic bacteria.—Kamen & Gest (44, 45) observed that *Rhodospirillum rubrum*, grown photosynthetically on a substrate such as malate and with glutamate or aspartate as a source of nitrogen, evolved H_2 vigorously in an atmosphere of He, A, or H_2. However, in an atmosphere of N_2 or an atmosphere of He in the presence of ammonia no H_2 was evolved. The influence of N_2 and ammonia on evolution of H_2 first suggested that a nitrogen fixing system might be present in the organism (45). As H_2 is a specific inhibitor of N_2 fixation by *Azotobacter* and the leguminous plants, its metabolism by an organism fixing N_2 assumes special interest. Gest & Kamen (30) have found that not only N_2 or ammonia but also high concentrations of yeast extract or peptone will suppress evolution of H_2. As ammonium salts and yeast extract have been used commonly in media for the growth of *R. rubrum*, it is not surprising that evolution of H_2 and fixation of N_2 were not observed earlier. Morita *et al.* (58) also have observed that the photoevolution of H_2 by *Rhodobacillus palustris* is suppressed by N_2, ammonia, or hydroxylamine.

Before the demonstration of Gest & Kamen, the only reported instance of biological photoproduction of H_2 was from adapted algae (28). However, Gest *et al.* (31) pointed out that the two processes are distinctly different, for evolution of H_2 from *Scenedesmus* (a) is not inhibited by N_2 but is by H_2; (b) is dependent on intracellular hydrogen donors; (c) appears only in adapted cells and is reversed by light; and (d) is much less active than evolution of H_2 from *R. rubrum*. Not only is the photoevolution of H_2 from the photosynthetic bacteria unique, but in the field of N_2 fixation it supplies the sought for but previously undiscovered example of inhibition of H_2 evolution by N_2. Although fixation of N_2 is inhibited by H_2, the hydrogenases found in species of *Azotobacter* (51),[4] *Clostridium kluyveri* (77), and *C. pasteurianum* (59) are not affected by N_2.

[4] Using ferricyanide as an acceptor Hyndman in our laboratory obtained evidence of inhibition of hydrogenase in *Azotobacter vinelandii* by N_2; Green, however, could not confirm this with the methylene blue system. It appears then that if N_2 did inhibit, it inhibited a carrier system rather than hydrogenase itself.

Early studies (31) indicated a high degree of substrate specificity in H_2 evolution, for only malate, oxalacetate, fumarate or to a limited extent pyruvate proved effective in supporting evolution of H_2 by *R. rubrum*. Kamen (44) reported that *Rhodopseudomonas gelatinosa* evolved H_2 in the light on a considerably wider range of substrates than did *R. rubrum*, but later, Siegel & Kamen (73) found that additional substrates also were effective for production of H_2 by the rhodospirilla.

During the early stages of the photometabolism of malate, 1 mole of CO_2 and 1 mole of H_2 are produced for each mole of malate used (10). In the presence of NH_4Cl or N_2 the production of H_2 is suppressed, but the moles of CO_2 released remain equivalent to the moles of malate consumed. The effect of ammonia in suppressing evolution of H_2 disappears immediately upon the exhaustion of the ammonia in the culture; likewise, replacement of N_2 with He in the atmosphere above the cells permits immediate resumption of H_2 release. N_2 has no effect on the uptake of NH_4Cl, for the cells apparently use the NH_4Cl preferentially as a source of nitrogen. The fact that the ammonium ion acts like N_2 in stopping the photochemical evolution of H_2 by *R. rubrum* suggests that ammonia may be formed from N_2 in the process of N_2 fixation (31); evidence derived from a study of the distribution of N^{15} in *R. rubrum* (93) points to the same conclusion.

Gest (29) found no evidence with inhibitors that a cell-free hydrogenase from *R. rubrum* was a sulfhydryl enzyme and no evidence for an easily dissociable cofactor on the enzyme when tests were made with methylene blue as the hydrogen acceptor. Ferricyanide would not serve as hydrogen acceptor for cell-free hydrogenase from *E. coli* or *R. rubrum*, but Hyndman *et al.* (37) found it a suitable acceptor for the cell-free hydrogenase from *Azotobacter*.

The products of the fermentation by *R. rubrum*, other than gaseous products and cells, long remained undefined. Kohlmiller & Gest (48) observed that the decomposition of pyruvic acid in the dark by normal resting cells closely resembles that effected by the propionic acid bacteria. Cells grown aerobically in the dark likewise ferment pyruvic acid to CO_2, H_2, acetate, propionate, butyrate, valerate, and caproate. In the light, no fatty acids are produced from pyruvate but only CO_2 and H_2 in equimolar amounts. Young cells produced formate anaerobically in the light on a malate-glutamate medium, whereas in earlier studies with old cells no formate was observed. This forced a reconsideration of the earlier interpretation that formate is an unlikely intermediate in production of H_2 and CO_2. Cells which have been furnished with formate will decompose it in the dark to CO_2 and H_2, but in a medium containing NH_4Cl sufficient to suppress liberation of H_2, formate does not accumulate.

Photoevolution of H_2 is not confined to the Athiorhodaceae, since several species of *Chromatium*, a representative of the Thiorhodaceae, also liberate H_2 from malate in the light but not in the dark (11, 61). Although the interrelationships of N_2 and H_2 metabolism in nitrogen-fixing bacteria remain obscure, an understanding of H_2 metabolism may well be of key importance in resolving problems of nitrogen fixation.

MECHANISM OF FIXATION

Probably no aspect of biological nitrogen fixation has been so thoroughly explored during the past decade as the chemical and enzymatic pathways followed by N_2. The spirited debates on various details of specific hypotheses have stimulated research in a number of laboratories with the rewarding consequences that today several fundamental steps appear to be fairly well worked out, and a general understanding of the over-all process from molecular nitrogen to cell protein is in prospect. Lest this view appears to be too optimistic, it can be claimed at least that sufficient knowledge is at hand to speculate intelligently on possible steps, and methods are available that can be used for experimental testing. Before documenting these conclusions, a statement regarding the nature of scientific "proof" may clarify just what is claimed and what is not.

Few scientists, certainly not biologists, believe that any one experiment, however crucial it may appear, can ever establish proof of a given hypothesis. Whether explicitly stated or not, most experiments are really designed to test some "null hypothesis" whose invalidation certainly cannot establish an opposing one, since seldom if ever does a unique alternative exist. The most that can be claimed for an experiment whose results are consistent with a postulated hypothesis is that it increases the probability of the correctness of that hypothesis; if inconsistent, the probability is correspondingly decreased. Obviously, the more results obtained in independent and diverse experiments that are consistent with a given hypothesis the greater becomes the probability of its correctness, but this probability may be still far from unity. Likewise, a so-called "negative result" does not necessarily invalidate a hypothesis but only decreases it toward zero.

With these limitations in mind, it is clear that at a given time only qualitative decisions clothed in such terms as "probable," "likely," "possible," "unlikely" can be made regarding an hypothesis. The specific one chosen will depend not only upon the results of the experiments made but also on their number and on the particular choice of the theoretical infinite number that might be performed.

In 1945 the authors (12) suggested a working scheme for research on the mechanism of nitrogen fixation which consisted of possible pathways from molecular N_2 (or nitrate) to amino acids. This scheme was somewhat more than a mere enumeration of theoretical compounds as it represented the various oxidation levels through which N_2 (or nitrate) must pass to become cell protein. We may expect, therefore, a compound corresponding to each of those illustrated in this scheme, not necessarily the one whose formula is given, but at least one of the same oxidation level. Actually other compounds must be added to make the scheme complete, since many of the steps are represented as two electron jumps whereas single electron transfers are more likely.

Ammonia as the key intermediate.—Experiments to date concerned with identification of postulated intermediates have dealt primarily not with the initial product of fixation but with the "key" intermediate, i.e., the com-

pound which represents the end of the fixation reaction and the start of assimilation of the fixed nitrogen into an organic carbon skeleton. For several years now the evidence strongly supports the view that this "key" intermediate is ammonia. It is emphasized that this view is based on results from many diverse and independent types of experiments made with the azotobacter, clostridia, photosynthetic bacteria, blue-green algae and the symbiotic system. The justification for combining experimental findings from diverse organisms and experiments has been discussed recently in an essay dealing with the comparative biochemistry of nitrogen fixation (99). Details of the experiments and typical results are contained in this publication; here we shall only summarize the evidence:

(a) Intensive studies extending over a period of five years in which N^{15} has been used as a tracer established that the pathway in representatives of all the major species of nitrogen-fixers was essentially similar as revealed by the distribution of the label among the various amino acids before equilibration of the isotope. The species studied included: *Azotobacter* (99), *Clostridium* (105); *Chromatium, Chlorobium*, and *Rhodospirillum* (93) and intact soybean nodules (103).

More recently, Magee (56) has determined the distribution of N^{15} in the cells of *Nostoc muscorum* supplied N^{15}-enriched N_2, nitrate, or ammonia for a short time. As with the other agents, glutamic acid had the highest level of N^{15} among the amino acids; aspartic acid also was high in N^{15}, the neutral amino acids were intermediate, and the basic amino acids were low in N^{15} concentration. The cells supplied N^{15}-enriched nitrate showed the least differential in their distribution of N^{15} but had notably high levels of N^{15} in their cytosine, guanine, uracil, and xanthine—levels as high as or higher than in glutamic acid. Other than for the high concentration of N^{15} in purines and pyrimidines, the distribution of N^{15} in the blue-green algae was much like that found in other nitrogen fixing agents. Also, it is of interest that among the free nitrogen compounds recovered from excised nodules exposed to N_2^{15}, the highest level of the label is found in glutamic acid (56).

(b) These same studies demonstrated that the distribution of N^{15} was quite similar whether $N^{15}H_4^+$ or N_2^{15} was supplied. The outstanding characteristic is the high level of the label found in glutamic acid; the shorter the time of exposure to the label, the higher the relative concentration of the label that appeared in this amino acid. These data suggest that molecular nitrogen proceeds through intermediate steps to ammonia which is then transformed into glutamic acid by reductive amination of α-ketoglutaric acid. The other amino acids are then formed by transamination reactions. Both glutamic dehydrogenase and transaminases have been found in species of *Azotobacter* and *Clostridium*, and undoubtedly examination would reveal their presence in the other nitrogen fixers, as both enzymes are widely distributed in nature.

(c) Ammonia immediately (i.e., without a period of adaptation) supplants molecular nitrogen when supplied to cultures actively fixing N_2. Under some circumstances the inhibition is complete, but it need not be, and the two sources can be used simultaneously, as would be expected of

an intermediate, as was noted by Zelitch using *C. pasteurianum* (102), and by Newton (60) using *A. vinelandii*.

(*d*) If the available supply of dicarboxylic acids is low, *C. pasteurianum* will excrete fixed nitrogen into the medium. Through isotopic trials these compounds were demonstrated to be "juvenile," i.e., recently fixed, since their content of the label was many times higher than that of cellular constituents (104). Isolation revealed asparagine, glutamine and ammonia. The N^{15} content in all was high, but the ammonia had by far the highest level; glutamine amide nitrogen was next. This isolation of intermediates provides the most direct and impressive evidence of the role of ammonia in the fixation reaction, a role already inferred from less direct evidence discussed in paragraphs *a*, *b*, and *c*. Newton (60) has recently provided an analogous demonstration with *A. vinelandii* using an isotopic dilution technique.

The role of hydroxylamine.—The immediate precursor of ammonia should be a compound at the oxidation-reduction level of hydroxylamine so it is not surprising that considerable speculation and experimentation has been directed toward the role of this compound. The specific question is whether the precursor is hydroxylamine itself or some derivative at the same oxidation level; this might be an oxime, hydroxamic acid or even a combination of hydroxylamine with an enzyme or coenzyme analogous to acetyl-CoA. The relevant experimental findings are:

(*a*) Originally, Virtanen considered hydroxylamine to be the key terminal compound in nitrogen fixation; at this point the fixation step was complete, and the inorganic nitrogen atom entered into the organic pool by combining with oxalacetic acid, which was then reduced to aspartic acid. The chief evidence was that under certain conditions aspartic acid is excreted from the nodules of leguminous plants. Arguments for and against the specificity of this finding (87, 97, 98) need not be repeated, since the isolation of glutamic acid among the excretion products has caused Virtanen to modify his original view so that now NH_2OH becomes either a precursor of ammonia or perhaps functions in an alternative pathway. Supporting data include the finding of oxime-N in cultures of nitrogen fixers (87, 91) and the preference of certain leguminous plants for aspartic acid in comparison with inorganic sources of nitrogen. The generality and significance of this latter support has been questioned by Ghosh & Burris (32) and Vantsis & Bond (86).

(*b*) From the point of view of comparative biochemistry, NH_2OH is practically a must in the reaction chain (99). It has long been regarded with favor as an intermediate in the reduction of nitrate by organisms (88), and recent studies (52) definitely established it as an intermediate in nitrification by the autotroph *Nitrosomonas*. Certain heterotrophic bacteria (*Nocardia corallina; Agrobacterium, Alcaligenes*) will convert the oximes of keto-acids into nitrite (39, 69). Because of the differential action of inhibitors on assimilation of nitrate and ammonia by *A. chroococcum*, Csaky (15) concluded that reduction of nitrate may proceed only to the hydroxylamine stage although isotopic dilution studies by Burris & Wilson (12a) established that ammonia is the terminal product of nitrate reduction by *A. vinelandii* O.

Recent investigations by Allen & van Niel (2) have cast doubt on many of
the views previously held about denitrification including the one that hy-
droxylamine is an intermediate.

(c) Enzymes for the metabolism of NH_2OH and its compounds exist in
many organisms including nitrogen-fixing bacteria. Cohen & Cohen-Bazire (14)
report that a species of *Clostridium* will reduce the oxime of oxalacetic acid
to aspartic acid[5], although Virtanen & Hakala (90) reject NH_2OH as an in-
termediate for this anaerobic agent. A more interesting possibility is that
suggested by the discovery of an enzyme that exchanges NH_2OH for the
amide ammonia of glutamine. Stumpf's (80) proposal that this enzyme may
be significant for biological nitrogen fixation certainly merits consideration
and experimental investigation. For example, NH_2OH could be detoxified
by exchanging with the amide group of glutamine; then the resulting hy-
droxamic acid could be reduced to glutamine and the fixed nitrogen be re-
leased as the key intermediate, NH_3, by repetition of the reaction. Note,
however, that the isotopic studies with *C. pasteurianum* already mentioned
hardly support this view since the highest label was in ammonia rather than
glutamine (104).

(d) The chief difficulty to be overcome is the readily demonstrable
fact that hydroxylamine itself is not utilized by nitrogen-fixing organisms
such as *Azotobacter* (66, 72) and *Clostridium* (71). Its extreme toxicity for
these organisms (65, 71, 72) suggests that this lack of growth on NH_2OH
does not arise from failure to penetrate the cell. Neither nontoxic levels of
NH_2OH (*circa* 3 μg. per ml.) nor oximes of the common keto acids are uti-
lized by *A. vinelandii* (72); *C. pasteurianum* will not grow on nontoxic levels
of hydroxylamine but will slowly use the oximes (71). Critical examination is
often difficult since hydroxylamine in aqueous solution decomposes into
NH_3 and N_2. Since the oximes dissociate into the base and keto acids, un-
equivocal interpretation of experiments is seldom possible even though they
last for only a few hours.

The initial product of fixation.—In recent years a start has been made
toward identification of the initial product of fixation. Although little direct
evidence either for or against the view exists, it is generally assumed that,
at least with the aerobic agents, the initial reaction is an oxidation. From the
point of view of comparative biochemistry an oxidized product appears to
be reasonable because of the similarities in properties of the reactions re-
sponsible for the reduction of NO_3^-, (NO_2^-) and of N_2 to NH_3. As hyponi-
trite (HNO) or its dimer has long been considered to be an intermediate in
the reduction of nitrate, this compound has been postulated as one of the
initial products of nitrogen fixation although not necessarily the first one.
Experimental evidence in support of this view, however, is not impressive.
The fact that N_2O is a competitive inhibitor for nitrogen fixation by *Azoto-*

[5] In a private communication Suzuki & Suzuki of the University of Nagoya
(Japan) have furnished us with similar results obtained with suspensions of *A.
vinelandii.*

bacter (70, 96) suggests that some compound at that level of oxidation occurs, but $(HNO)_2$ itself appears to be unlikely since, like NH_2OH, it is not utilized in nontoxic concentrations by either *C. pasteurianum* (71) or *A. vinelandii* (96). Moreover Allen & van Niel (2) rejected both this compound and its anhydride (N_2O) as intermediates in denitrification by this organism. The compound at the hyponitrite level of oxidation was identified as nitramide.

Since hyponitrite was invoked primarily from comparative biochemical considerations, its elimination as an intermediate in denitrification might cast serious doubts on its participation in nitrogen fixation. We have attempted to test the utilization of nitramide but have been unsuccessful to date because the crystalline compound prepared in our laboratory (by M. M. Mozen) decomposes in a few minutes when added to either Burk's medium or buffer solution of physiological pH. Perhaps utilization or non-utilization of N^{15}-labeled nitramide can be established despite its instability in solution.

Two other types of studies are significant in the search for initial products of fixation. Although some disagreement still exists as to whether the hemoglobin found in root nodules of leguminous plants is of importance as a carrier of O_2 at the low pO_2 within the nodule (13, 76), all would probably agree that this does not preclude direct participation of this compound in the fixation reaction. If direct participation is established, then the existence of an initial oxidizing reaction becomes much more probable. Several workers have now reported isolation of natural and induced mutants of *Azotobacter* species unable to fix nitrogen but which will grow on NH_3 (34, 46, 101). Although most of these mutants readily revert to the wild type when kept on a nitrogen-free medium, many persist long enough for physiological experiments to be made (33). Such mutants could be quite useful in the detection of compounds occurring betwen N_2 and NH_3.

PERIPHERALIA

A common experience of reviewers is to finish the manuscript with several unused references. These range from articles that might have been included in the material discussed, but were omitted for various reasons, to scientific *curiosa* whose relevancy is vague. The easy decision to forget them often denies the reader access to diverting bits of knowledge. To avoid this, we have supplied this section of somewhat unrelated topics that appealed to the reviewers.

For several years Fedorov has published numerous papers in support of a hypothesis that the structure of the nitrogen-fixing enzyme in species of *Azotobacter* included —COOH, —NH$_2$, and —CO groups. The carbonyl group after hydration reacts with N_2 to form the linkage —C:N—NH$_2$ with the liberation of oxygen. This hydrazine derivative passes into the protein of the cell and can produce NH_3 and NH_2OH as by-products (22, 23). The experimental support is primarily based on growth experiments using a great variety of substrates (24) in the presence of various inhibitors such as

ethylurethan, diphenylurea, alphatic alcohols, and inorganic salts. Among
other claims are: that a hemin compound does not directly participate in the
reaction (23, 25); that the active surface developed in the fixation of 1 mg.
N is approximately 2500 m.^{2} (26); that the nitrogen-fixing system is insepa-
rable from living tissue (contrary to the claim of his countryman, Bach) (26);
and that even 0.1 M oxalate ion will not prevent nitrogen fixation but does
completely inhibit development of the organism and oxidation of the carbon
source (25).

These sweeping claims are at such variance with many widely accepted
views that independent verification and interpretation of the results are
needed. The reviewers do not qualify to pass judgment on the mass of ex-
perimental data and conclusions since they have seen nearly all of the ma-
terial only as abstracts (the cited references are just a fraction of the many
articles appearing in the Russian literature that were summarized in *Chemi-
cal Abstracts*). Examination of the original in two instances (22, 26), how-
ever, revealed that the experimental methods employed were not likely to
yield profound answers to the questions asked. The growth experiments in
both shaken and quiescent cultures lasted from 6 to as many as 30 days; it is
not surprising that the efficiency (mg. N fixed/gm. sugar used) was only
about one-half that routinely obtained in modern investigations, e.g. in
short-time manometric trials or even in 24 hr. experiments using shake
flasks. Although, as we have indicated, much work can still be done in this
field using simple, traditional methods, we do not believe that informed
studies on the mechanism are included. Long time growth experiments were
justified when no other technique was available, but they became dated with
the introduction of the first modern tool [the manometric method (97)] for
investigation of this biological mechanism. Answers for the unsolved prob-
lems discussed in the preceding sections will probably depend on develop-
ment of still more powerful methodology rather than a return to one that has
long since served its purpose.

Biological nitrogen fixation has never lacked material for controversy
ranging from polite disagreement to acrimonious debate. Among those stirring
present day workers are the alleged symbiosis between nitrogen-fixing or-
ganisms and animals and the role of weather in affecting the fixation reac-
tion. Toth's report that insects could fix nitrogen (12) became less surprising
when the fixation was ascribed to organisms, including species of *Azotobacter*,
living in the tissues (83). The claims have now been extended to include
higher animals although even Toth (82) admits that the rumen of a goat is
not a likely place for fixation to occur because of its high nitrogen content.[6]
Other workers (49, 75) have disputed the significance of the observations
even for insects. The proposal, apparently seriously made, that biological
nitrogen fixation is a factor in the sounding of whales [see (99) for early
literature] still lacks experimental and logical confirmation (38).

[6] Toth's present views and the evidence in support have been summarized in two
recent publications (83 a and 83 b).

In spite of Jensen's negative findings (40), Bortels reiterates his belief that nitrogen fixation by the *Azotobacter* is controlled in part by a weather factor, particularly the barometric pressure. In recent summaries (8, 9) he discusses this as one aspect of his general theory dealing with the effect of weather on various biological phenomena, including pigmentation of bacteria, germination of spores, and host-parasite relationships. The reviewers do not wish to deny categorically that weather affects nitrogen fixation, because apparently it does play a role in events such as the excretion by nodules of leguminous plants (97) and certainly in the relationship between photosynthesis and nitrogen fixation (54). For many years, however, we have routinely measured nitrogen fixation by *A. vinelandii* O under carefully standardized conditions that insure a high efficiency. The outstanding characteristic has been the consistency of the results in aeration bottles, shake cultures and in manometric trials (15 to 20 μg. of N-fixed/mg. of sucrose depending on the particular experimental conditions). Efficiencies less than these, accompanied by wide variations in total fixation, when investigated have always turned out to be caused by nothing more bizarre than the usual ills of the biochemical laboratory: contaminated cultures, improperly prepared medium, or faulty aeration. Conclusions based on lower efficiencies and rates of fixation (about 10 μg. N/ml. in 18 to 24 hr.) are open to suspicion since so many factors are limiting the process that it is difficult to single out the one responsible for an observed variation.

Erkama & Nurmikko (19) were unable to confirm Dhar's claim [see (12)] of nitrogen fixation induced by light, but Virtanen & Ellfolk (89) from the same laboratory describe another type of nonbiological fixation, that induced in an ultrasonic field. Of interest is their observation that both H_2 and CO, but not A inhibit the reaction analogous to the effect of these gases on aerobic, biological nitrogen fixation.

Although, as has been already indicated, the precise role in nitrogen fixation of the hemoglobin found in nodules of leguminous plants is still undetermined, continued investigations on details of its structure (18), the manner of its occurrence in the development of the plant (43, 62), as well as its function, if any, in respiration (13, 76, 85) are making significant progress toward this goal. It is perhaps fitting that this section should be closed with a reference to the interesting study in comparative physiology by Klüver (47) which is concerned with possible use of root nodules for research in neurology and psychiatry. The relationship arises through a metabolic disease, acute porphyria, which can lead to a variety of nervous and mental symptoms. The occurrence of a free porphyrin-hemoglobin system in root nodules may provide useful experimental material for study of the system in the absence of factors that influence the interrelations between porphyrins and hemoglobin in the animal. A great deal of chemical work on the system in nodules of the soybean is described, results that might be overlooked since the *Journal of Psychology* is not usually consulted by research workers in biological nitrogen fixation.

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INDUSTRIAL FERMENTATIONS[1]

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Although no general review under this heading has appeared in the *Annual Review of Microbiology* since 1947, the related topics of metabolism, nutrition, and genetics of microorganisms have been dealt with almost every year since then. Beginning in 1948, a comprehensive review on fermentations has been compiled each year by Lee and his associates (116, 117, 118, 158). In these reviews particular emphasis was placed on fermentation as a unit process. No attempt will be made here to cover this aspect of the subject; rather attention will be focused primarily on publications which have contributed to advances in production of the more important fermentation products. Recently an excellent general paper on microbial metabolism and its industrial applications was presented by Kluyver (110) to the Royal Institution in London.

Antibiotics

The literature on antibiotics has become so voluminous that it is quite impossible to mention other than a few of the more significant developments of recent years. Several noteworthy text books have been published, of which two volumes by Florey and colleagues (68), Waksman's *Streptomycin* (219), Baron's Handbook (11), and Pratt & Dufrenoy's treatise (167) might be mentioned. Some general reviews are those of Raper (173), Brian (25), Robinson (176), and Lumb (132).

Penicillin.—Many factors have contributed to the great increase in efficiency of penicillin fermentations in recent years. These have been reviewed by Peterson in the Harvey lectures (163) and Perlman (156). In numerous laboratories selection of cultures and mutants led to the isolation of high yielding strains with desirable characteristics. The work of Backus *et al.* (10), produced the now famous strain of *Penicillium chrysogenum*, Wisconsin Q176, through ultraviolet irradiation. Woodruff & Larson (232) patented the development of pigmentless strains which not only outyielded the parent Q176, but gave higher percentages of penicillin G.

Formulation of media, especially the addition of intermediates or precursors, has been studied more intensively than any other aspect of this fermentation. The addition of phenylacetic acid, or derivatives of it, greatly increased the proportions of benzylpenicillin which is still the most important biosynthetic type produced. In these studies Johnson and his students have made many important contributions (101, 142, 187, 207), as have the research staffs of Lilly (15, 16, 17, 18, 19), Abbott (166), Glaxo (127), and Hoffman-Laroche (201). Quantitative studies have also been made on the

[1] The survey of the literature pertaining to this review was concluded in February, 1953.

phosphorus, sulphur, potassium, magnesium, iron, and copper requirement of *P. chrysogenum* Q176 for growth and penicillin production (102). Numerous studies have been conducted on the carbon and nitrogen sources, together with pH control and maintenance of adequate aeration and agitation. Because these factors particularly affect industrial production much of the work has never been published, although a survey of the patent literature reveals many of the basic improvements effected. Soltero & Johnson (192) recently have completed studies on carbohydrate nutrition and penicillin production. Both glucose and sucrose gave better yields than lactose if fed at 12 hr. intervals. Corn steep liquor still plays an important role in most media, although there have been many attempts made to replace it or enhance its effects through the addition of amino acids (145), esters of long chain fatty acids (38), or of vegetable oils. Initially oils were used to control foaming, but it was soon found that they affected fermentation yields apart from their foam depressing qualities. Calam *et al.* (29), noted the effect of various fatty acids on respiration and growth of the fungus during penicillin production. For other studies of the factors affecting this fermentation as well as much fundamental information on other submerged fermentation processes the reader should consult the series of papers presented to the American Chemical Society meeting at Atlantic City in 1949 (12, 13, 26, 27, 72, 85, 197).

Streptomycin.—In comparison with the vast amount of effort put into obtaining high yielding penicillin fermentations, streptomycin moved very rapidly from laboratory into full scale industrial production using the submerged process. Mutants produced by ultraviolet, x-ray, and nitrogen mustard have all contributed to the making of high yielding strains of *Streptomyces griseus*. Waksman (220) conducted a very interesting study on the production of a streptomycin-producing mutant from one of his cultures originally isolated in 1915. During thirty years of subculturing this isolate apparently had lost its ability to produce streptomycin. On irradiation a mutant was selected which was the equal in streptomycin-producing ability of new strains isolated in 1943. Stanley (195) was able to increase yields of from 100 to 200 μg. per ml. to 400 to 500 μg. per ml. through strain selection alone. After irradiation of this culture with ultraviolet light, colony selection yielded cultures which produced 600 to 800 μg per ml., one strain exceeding 900 μg per ml. in shake flasks. The response of the mutants differed widely depending upon media used and type of fermentor. More recent studies using ultraviolet light, x-ray, or nitrogen mustard are those of Savage (178) and Dulaney *et al.* (53). Dulaney lists many morphological and color changes in the colonies but was unable to correlate any of these with streptomycin production. A patent issued to him in 1951 (55), claims production of new strains of *S. griseus* capable of yielding titres of antibiotic in excess of 800 units per ml. Such strains must be selected on the basis of high resistance to high initial concentrations of streptomycin.

Improved media have also been responsible for increased yields. The cost of production has been lowered through substitution of cheaper nutrients,

such as mixtures of corn steep liquor, soybean or cottonseed meals, or distiller's solubles, for animal proteins or protein hydrolysates. Most of these improvements are mentioned in the patent literature.

Thornberry & Anderson (208) studied the nutrition of *S. griseus* in synthetic media and determined the essential requirements. Contrary to earlier reports they found that nitrate and nitrite could not be utilized by the organism. Dulaney (51, 52) has also studied both nitrogen and carbon sources in some detail and agrees with the above findings concerning nitrate and nitrite. He also found that of all the amino acids tested L-proline was the only one that gave yields as high as 800 μg per ml., when used as a sole nitrogen source in his synthetic media. However, no increase in yield was observed if ammonium nitrogen was present. In general, organic acids were poor carbon sources, as were also pentoses. The best hexose sugars were glucose and mannose.

Aeration and agitation are other critical factors which have been studied. Investigators in the Merck laboratories (12) measured the mass transfer of oxygen in submerged fermentation by *S. griseus*. Wise (231), using this organism, has also made an excellent contribution towards more precise measurement of the aeration of culture media.

Biosynthetic radioactive streptomycin has been produced by Karow *et al.* (108). Labelled glucose provided the best substrate since it appeared to be preferentially utilized for streptomycin formation.

Actinophages appeared to plague the producers of streptomycin very early in the course of production. Koerber *et al.* (112) showed that two types of phage were present, and they found that resistant strains of *S. griseus* could be readily isolated. Phage may also be controlled, at least partially as shown by Perlman *et al.* (157), by addition of substances sequestering calcium in the medium, provided that it is done before infection of the host occurs. Citrate, oxalate, and phytate are also effective for inhibiting phage in submerged fermentations without affecting yields of the antibiotic.

Like penicillin, streptomycin as produced by various strains of *S. griseus* or related species is not always a pure chemical entity. It was discovered by Titus & Fried in 1947 (209), while fractionating crude streptomycin preparations, that mannosidostreptomycin occurred therein. This compound was shown by the Squibb Workers (114) to be a natural streptomycin which is produced early in the course of fermentation, later being converted to streptomycin by an enzyme system. In 1950, Benedict *et al.* (21) discovered a streptomycin in which the streptose group contained an extra oxygen atom. Grundy *et al.* (75) independently discovered the same compound and a year later reported that the organism producing it resembled the new species, *Streptomyces griseocarnus*, reported by the Northern Regional Laboratory in Peoria. Hydroxystreptomycin, as this compound is now called, possesses antibacterial activity and pharmacological properties very similar to streptomycin and probably has no advantage over it as a chemotherapeutical agent. The workers in the Abbott laboratories (164) have studied its production

in shake flasks, 30 l. stir jars and 400 gal. fermentors, and moderately good yields were obtained. A good general review of streptomycin, its uses, properties, and production was published recently by Emery of the Glaxo Laboratories in England (61).

The broad spectrum antibiotics.—Three antibiotics, chloramphenicol, aureomycin, and terramycin, unknown when the last review on industrial fermentations in this series appeared in 1947, are now being produced on a large industrial scale by different companies. All are produced by species of *Streptomyces*, and while these antibiotics differ chemically, they have much in common therapeutically. Numerous articles have been published in recent years on their chemistry, biological properties, and role played in medicine, but very few details concerning their production are available. The basic processes are covered in each case by patents belonging to the companies responsible for their discovery (57, 60, 191).

John Ehrlich and co-workers of the Parke, Davis & Co., together with Burkholder of Yale University (59), announced their discovery of chloramphenicol in October, 1947. About the same time, Gottlieb and co-workers (59) at the University of Illinois isolated the same antibiotic, and the two teams published a joint paper naming the producing-species, *Streptomyces venezuelae* N. Sp. Evidence was presented showing that *S. venezuelae* was a new species and distinct from cultures of *S. lavendulae* which it most resembled. In the original paper (58) yields of chloromycetin of 85 μg. per ml. were reported in 30 l. glass fermentors, using submerged cultures in medium containing maltose, casamino acids, distiller's solubles, and sodium chloride. Additional biological properties of chloramphenicol were described by Smith *et al.* (189) the same year, but since then, possibly owing to its successful chemical synthesis, further developments in the fermentation process have been slow in appearing. In 1951, Gottlieb & Diamond (73) reported success in creating a synthetic medium which gave yields of chloramphenicol equal to those from complex organic substrates. A general outline of the biological process used for the manufacture of chloramphenicol appeared in 1949 (150). It has been shown (152) that, in contrast to penicillin, the period of most rapid biosynthesis occurs at the time of mycelium formation.

The discovery of aureomycin was announced by Duggar and associates in July, 1948 (see 56). Duggar has briefly described the organism, *Streptomyces aureofaciens*, as a new species first isolated from soil collected in Missouri. Both the mold and the product derive their name from the golden yellow pigment which is present in the mycelium, the medium, and finally the antibiotic. Aside from three patents on biological production, recovery, and further purification, the only paper of importance concerning production is by Van Dyck & De Somer (218). Starting with a streptomycin medium they investigated various carbon and nitrogen sources and found that commercial sucrose and peanut oil meal or peanut meal with a low fat content gave the best yields. A medium on which they obtained yields of 1300 μg. per ml. was developed. The basic strain of *S. aureofaciens* was improved by strain selec-

tion and ultraviolet irradiation resulting in new isolates yielding about three times the product of the parent strain.

Terramycin was first announced by Finlay *et al.* in January 1950 (66). It was discovered during a comprehensive soil screening program conducted in the laboratories of the Charles Pfizer & Co. The first detailed publication followed a conference on the antibiotic conducted by the New York Academy of Sciences in June 1950 (106), disclosing biological properties, pharmacology, and clinical results. No details of the organism or the fermentation were presented. The medium cited for production in the terramycin patent (191) contained soybean meal, corn starch, N.Z Amino B, $NaNO_3$, $CaCO_3$, and vegetable oil to control foaming. The fermentation is carried out in large aerated fermentors. A flow sheet of the Pfizer process, together with pictures of parts of their plant and a very general outline of the steps leading to production of this antibiotic appeared in January 1952 (174).

A fourth broad spectrum antibiotic, which has just recently come on the market was announced in June 1952, by McGuire *et al.* (139) of the Lilly research laboratories. It is produced by a strain of *Streptomyces erythreus* isolated from soil collected in the Philippines. When the organism was grown on a soybean meal-glucose medium in shake flasks the broth was active against gram positive bacteria and mycobacteria. Animal and chick embryo tests showed activity against typhus rickettsiae, and certain large viruses. The crystalline antibiotic was isolated and given the trade name "Ilotycin." In the editorial introducing this antibiotic (222) the name "Erythromycin" was used, and some very interesting figures on production of the major antibiotics during May 1952, were cited as follows: crystalline penicillin 30 tons, streptomycin and dihydrostreptomycin 21 tons, the broad spectrum antibiotics 24 tons. This is, indeed, a remarkable development, for only a decade ago the production of penicillin was just beginning and the others were unknown.

Miscellaneous antibiotics.—Raper (173) has estimated the number of antibiotics now known to be approximately 300 of which 147 come from fungi 86 from bacteria, 73 from the actinomyces, and the remainder from seed plants, lichens, yeasts, algae, and miscellaneous sources. Apart from the five major antibiotics already mentioned, none have achieved major importance. Some are in limited production and are finding use, mainly, for topical applications in human medicine, control of animal and plant diseases, insect pests, amoebal diseases, poisons for rodents, and as feed supplements.

Perhaps mention should be made of a few of the bacterial antibiotics. Recently a plant was built for the production of bacitracin which is finding a market as a feed supplement, particularly for young pigs. This antibiotic was originally described by Johnson *et al.* (104) in 1945 from an unidentified *Bacillus* sp. which is probably *B. licheniformis*. In 1949 the same authors described methods of production, concentration, and partial purification with a summary of the chemical properties of the crude antibiotic (6). Three other antibiotics which have received much attention are tyrothricin, sub-

tilin, and polymyxin. The latter, discovered independently by three groups of workers in 1947, at the Northern Regional Laboratory (20), the American Cyanamid Company (196), and the Wellcome Laboratories (4) in England, has proved to be a complex group of at least five polypeptides now designated polymyxins A, B, C, D, and E. Numerous papers have appeared on the production, chemistry, and pharmacology of these compounds.

Probably the most intensive search for new antibiotics made during recent years has been among the actinomycetes. Many streptomyces are capable, depending upon media and conditions used in the fermentation, of producing several unrelated compounds simultaneously. For example, *S. griseus* produces grisein, streptomycin, mannosidostreptomycin, streptocin, and cycloheximide, whereas *S. lavendulae* produces streptolin and streptothricin. In contrast *S. fradiae* produces a complex of several chemically related substances known as the neomycins A, B, and C. Those interested in the physiology of this group of microorganisms and a review of their manifold biochemical properties, with special emphasis on antibiotic production, should read the recent review by Perlman (160).

In spite of many surveys on different groups of fungi, few genera, with the possible exception of *Aspergillus* and *Penicillium* have been adequately surveyed for production of antibiotics. Brian, in his review of the fungi (25), emphasized the spotty treatment accorded many families. He mentions 96 different antibiotics of which only 57 have been obtained in pure form and are well characterized. Three properties, common to most of these products are their marked lipoid solubility, low water solubility, and their general toxicity. Whether or not other antibiotics as generally useful as penicillin still lie hidden, there is little doubt that further research will uncover many remarkable new compounds even from well known fungi. As an example, here in the Prairie Regional Laboratory, Haskins (80) noted that cultures of *Ustilago zeae*, the causal agent of corn smut, exhibited antagonism to certain fungi and bacteria. Submerged aerated cultures of this pathogen grown in a simple glucose-urea medium produced remarkable yields of long needle-like crystals which exhibited antifungal and antibacterial activity (81). Lemieux (121) has shown that these crystals are a mixture, composed essentially of two closely related D-glucolipids, which have been named ustilagic acids A and B. Although nontoxic to animals, this substance has not appeared promising as an antibiotic, possibly because of its high insolubility. It may, however, prove valuable as a starting material for preparation of macrocyclic musks used in perfume compositions (122).

ALCOHOLS

Synthetic alcohols are likely to provide increasing competition in the future, except in countries where cheap carbohydrates are available or the chemical industry is not far advanced. In Canada butanol is produced synthetically, whereas ethanol production from fermentation of waste sulphite liquor now appears to be firmly established. In the United States the per-

centage of both of these chemicals produced by the fermentation industries is declining.

Ethanol.—The best review which has appeared on industrial alcohol in recent years is that of Jacobs (100). In it he summarizes the technology, production, and uses of alcohol in relation to agriculture and cites most of the pertinent literature appearing in the past quarter century. Another general article on the subject, outlining the American position in 1951, was compiled by the editors of *Chemical and Engineering News* (35). Those interested in developments taking place in Britain on the production of industrial alcohol from molasses will find an interesting review by Dawson (48).

Following the intensive studies made on the processing of wheat prior to 1947, other sources of starch have been receiving attention. Arnold & Kremer (8) analyzed the position of corn as a potential raw material source and concluded that unless the price dropped to 40 to 60 cents per bushel or lower, while other prices remained unchanged, corn was uneconomical. In the United States they thought that ethylene and molasses alcohol, with minor amounts from sulfite liquor, grain, hydrocarbons via the Fischer-Tropsch synthesis, and possibly wood waste would continue to meet the demand. A comprehensive review of most of these processes is presented along with their studies on corn. In 1951 Kolachov & Nicholson (113) reviewed carbohydrate sources for ethyl alchohol production, stressing the role played by the distilling industry in enhancing the over-all value of the nation's grain crops. Kolachov, with other members of Joseph E. Seagrams & Sons staff at Louisville have been also assessing the potentialities of several tropical raw materials, including cassava from Brazil (206) and *Bassia* flowers from India (133).

Alcohol production from waste sulphite liquor of the pulp and paper industry appears to be firmly established in North America, with two firms operating in Canada and one in the United States. It is interesting to note that the large plant built at Gatineau, Quebec, began production during a period when alcohol production from either molasses or cereal grains was having difficulty competing with the synthetic product. No really detailed technical description of the process employed in the above plant has been published, apart from a general outline in 1952 (7). The Forest Products Laboratory at Madison, Wisconsin, has continued studies on the fermentation of wood hydrolyzates. Although much of the work has been directed toward production of food yeast, Harris and co-workers (78) used a strain of *Torula utilis* for the production of alcohol from Douglas fir hydrolyzates. The strain they were using, after acclimatization, was more satisfactory for the production of alcohol from wood hydrolyzates than brewer's yeast because of its resistance to inhibiting substances.

In general, the alcohol fermentation industry still uses batch processes for production, although several continuous processes have been described. Following the original work of Unger at Seagram's ten years ago, Ruf *et al.* (177) described a continuous process using acid-hydrolyzed grain mashes in

both laboratory and pilot plant in a 12 hr. cycle. The process described overcame two major problems of continuous fermentation by using a sterile mash in which the starch was completely hydrolyzed. However, it was necessary to add either wheat bran, mold bran, commercial amylase preparations, or submerged amyolytic mold culture to the medium to obtain high yields.

Much work has been carried on in recent years on the production of fungal amylases in submerged cultures. Part of these advances will be discussed in the section on enzymes. In a review of the various processes, Jackson *et al.* (98) indicated substantial savings in operating costs, using fungal amylase rather than malt in grain fermentations. However, the work of Pan *et al.* (154) indicates that submerged fungal cultures convert an appreciable portion of starch into a dextrin which is not further hydrolyzed easily.

Butanol-acetone.—In spite of intensive studies which have continued ever since World War I in different parts of the world, one receives the impression that many problems relating to this complex fermentation remain unsolved. Theoretically it should be possible to control the ratios of the different solvents produced by additives to the media, but little success appears to have been achieved over the years. Acetone yield may be increased by addition of nitrate nitrogen, but control of butanol yields apparently has not been accomplished. Those interested in good general discussions of some of these problems, as well as basic information, should consult the papers of Gill (71) and Beesch (14). The latter paper presents a comprehensive list of organisms which have been patented for use in this type of fermentation. Beesch also stresses the problems which arise in connection with bacteriophage in the industry.

Recently several papers, with much detailed experimental data have been published by the United States Forest Products Laboratories, the Northern Regional Laboratories, and the University of Wisconsin, concerning the practicability of using wood sugars obtained through acid hydrolysis (124), waste sulphite liquor (229), and the xylose saccharification liquors from corncobs (115). In general many difficulties are encountered and, while moderately good fermentation may be obtained, yields are relatively low.

Polyhydric alcohols.—Although no fermentation industries operate at present for the production of the higher alcohols such as glycerol or 2,3-butanediol (2,3-butylene glycol), brief reference might be made to a few of the recent papers on these chemicals. In recent years the expansion of synthetic glycerol and glycol production, especially ethylene glycol, has discouraged further developments with fermentative processes. However studies on their microbial production have contributed to our knowledge of fermentation processes and are therefore of general interest. The literature prior to 1949 has been reviewed by Prescott & Dunn (168), Underkofler & Fulmer (215), and Ledingham & Neish (95). Since 1949 a number of papers have appeared from the Ottawa and Saskatoon laboratories of the National Research Council of Canada. Pilot plant studies on the fermentation of wheat (22), recovery of levo-2,3-butanediol from wheat mashes (224), and

fermentation and recovery studies with barley mashes (210) indicated the feasibility of commercial production from cereal grains. Australian investigations on the *Aerobacillus polymyxa* fermentation of wheat were published by Crewther (42, 43, 44) in 1950. He found the addition of lactose, whey, or molasses to increase the sugar content of the wheat mashes resulted in undesirable effects. The addition of sucrose, however, increased the yield of 2,3-butanediol. The high cost of these raw materials has discouraged further development. The Ottawa laboratories have since investigated cheaper raw material sources such as waste sulphite liquor (146) and sugar beet molasses (147, 185).

Recently various fermentation processes have been studied for the production of glycerol. Underkofler & Fulmer, with their students, have reinvestigated the addition of sulfites and other compounds to yeast fermentations, using glucose (216), disaccharides, and starch (217) as raw materials. The addition of excess magnesium sulfite, followed by massive yeast inoculation, gave satisfactory yields using glucose, black strap molasses, high test molasses, sucrose, and acid hydrolyzed corn or wheat starch provided the pH level was maintained at 6.0 to 6.5. Fermentation of maltose in the presence of sulfites was very sluggish, hence enzyme-converted grain mashes were unsatisfactory. Neish & Blackwood (148), while conducting yeast fermentations at poised hydrogen ion concentrations, noted that the yield of glycerol was effected by the initial glucose concentration, the pH, and the presence of ammonium hydroxide. The highest yield of glycerol they obtained was 29 per cent by weight of the sugar fermented.

Since the original discovery of the glycerol-2,3-butanediol fermentation by our group in 1945, attempts have been made to gain a better understanding of this process. In spite of intensive studies with different strains of *Bacillus subtilis* (Ford type) it has been impossible to obtain high yields consistently. Blackwood & Simpson (23) have studied factors affecting the fermentation, but the process still remains quite unsatisfactory from the industrial viewpoint.

ORGANIC ACIDS

Molds and bacteria produce a wide variety of organic acids but, other than citric, lactic, acetic, and gluconic acids, few have ever attained importance in the fermentation industries. There is little doubt that with intensive strain selection, plus irradiation or chemical treatments of the organisms to induce mutant races, yields of many of the compounds could be greatly increased. Citric acid remains one of the most important chemicals produced by fermentation. There is evidence that this industry is now profiting from the many improvements, particularly in submerged mold fermentations, worked out for the antibiotics. With new plants coming into operation, and widespread knowledge of the techniques of production, competition is likely to be of more importance than previously.

Citric acid.—Perlman (155) has recently described the general procedures

used in both shallow pan and submerged processes, with particular emphasis on selection and maintenance of cultures and selection of media. *Aspergillus niger* is still the organism preferred for use in the citric acid industry, but one cannot help wondering whether a real effort toward selecting and improving strains of other species might not be worth while. In this connection it is interesting to note that the first paper to appear during the period under review was concerned with *A. wentii*, not *A. niger* (107).

Investigations at the University of Wisconsin were carried out with *A. niger* in shake flasks, and in a nine day fermentation as much as 72 gm. of anhydrous citric acid were obtained from 100 gm. of sucrose (181). The same authors previously had found that carry-over of manganese in the spore inoculum could be detrimental to yields obtained in the submerged cultures (180). Shortly after the war, in the National Research Laboratories at Ottawa, investigation began on shallow pan and submerged production of citric acid from beet molasses. Preliminary work with ferrocyanide treatments (37) in shake flasks and Kluyver flasks aerated with oxygen appeared encouraging. More recent work by Martin & Waters (134) in tower-type fermentors, using ferrocyanide-treated beet molasses, has resulted in greatly increased yields and reduction of time required to six days. An average rate of 0.93 per cent conversion per hour was obtained.

The Northern Regional Laboratory has also been investigating citric acid production. Moyer (143, 144) has introduced a novel feature in the addition of ethanol or methanol to stimulate production of citric acid by *A. niger*. Higher levels of zinc, iron, and manganese appear to be tolerated in either surface or submerged cultures if a slightly toxic concentration of either alcohol is present. Although they have not published their results, there is no question that many industrial laboratories have been investigating the deep tank fermentation process for citric acid production in recent years. The Miles Citric Acid Plant is now in production (34), presumably based on the results disclosed in two U.S. patent applications (179, 233).

Minor element nutrition has long been known to play a very important role in this fermentation. In recent years the ion-exchange or chelating agents have proved most useful in purifying medium components, thus making well controlled experiments possible. Tomlinson *et al.* (211, 212) and Chesters & Rolinson (36) have contributed to our knowledge of the role played by zinc, iron, copper, and manganese in metabolism of *A. niger* and production of citric acid.

Aeration and oxygen tension of the medium are undoubtedly of prime importance in citric acid production. To date no one has published detailed work comparable to some of the studies made for antibiotic production, but Erkama *et al.* (63) of Finland, have shown that aeration over surface cultures of *A. niger* nearly trebles oxalic acid formation while decreasing citric acid production to one-fourth. They attribute this reduction in citric acid to the sweeping away of the CO_2 evolved during respiration, thus affecting the Wood-Werkman reaction. Using different oxygen pressures Erkama &

Hägerstrand (64) demonstrated that at low levels iron accelerated acid formation but at high oxygen levels the best yields of citric acid are obtained in iron-free nutrient solutions.

It would be useful in studying aerobic fermentations of this type to be able to construct accurate carbon balances. Recently Shu & Thorn (183) of this laboratory worked out a procedure which provides an accurate material balance.

In India, Damodaran & Rangachari (47), through use of various enzyme inhibitors, have contributed to a better understanding of the enzymatic processes involved in citric acid formation. Recently, in a number of laboratories, radioactive tracers have been used to elucidate the mechanism of citric acid formation (31, 130, 131, 135). Walker (221) has written a general review on the pathways of acid formation, dealing fully with *A. niger* and citric acid production.

Lactic acid.—Lactic acid is becoming increasingly important as a basic chemical in the manufacture of plastics and other products in addition to its conventional use in the food industries. In recent years direct chemical production from sucrose has been improved and this process ultimately may compete with the fermentation processes. However, the fermentation has been successfully adapted to use cheap raw materials such as waste sulphite liquor. Leonard *et al.* (125) found that *Lactobacillus pentosus* would ferment the sugars present if the liquor was first steam stripped and the sulphite precipitated out at pH 8.5. Amyl alcohols and isophorone were good solvents, and the acid could be recovered readily by extraction procedures. Apparently the pH has not been automatically controlled during fermentations of waste sulphite liquor, but, judging by the marked improvements in rate of production and yield found by University of Minnesota workers on wheat grit or dextrose media (67, 109), further improvements might be expected.

At the Eastern Regional Research Laboratory, Cordon and associates (41) have continued their studies on the use of cull potatoes as raw material. Amylase of *A. niger* was used to saccharify the starch, after which species of *Lactobacillus* were used for acid production. *L. pentosus* gave the highest yields.

As with citric acid the mechanism of lactic acid production recently has been the subject of several studies. Gibbs *et al.*, (70) using C^{14} labelled glucose fermented by *L. casei*, found evidence that a partial randomization occurred between carbon atoms 1 and 3. Changes in the fermentation conditions did not shift this distribution of activity. Carson *et al.* (30) used a species of *Rhizopus* and, contrary to older views that aeration is detrimental, they found that O_2 is used directly in the process of lactate biosynthesis.

Miscellaneous acids.—There are several organic acids, including itaconic, fumaric, and kojic, which could be produced by fermentation if demand for them develops. However, these will not be reviewed here, nor the various keto acids which have been receiving increasing attention of late.

The production of acetic acid for the food industries is still an important

industry, and it is interesting to note that some research is being directed toward making improvements in the process. In producing a food product where flavor is of importance, achievement of high yields is not always the only consideration. Frequently the source of water used in the process may have an effect on quality. An interesting study on the effect of various types of dilution water used in vinegar manufacture has been made by Allgeier *et al.* (5). Comparison of water samples from widely scattered regions in the United States indicated that the more desirable ones were low in solids, had little chloride, and were not too hard. The authors also stated that further studies are needed on trace metal content, ionic balance, and degree of chlorination, as well as bacteriological purity. Another timely study has been to compare the efficiency of the conventional shaving-packed towers with the new aerobic submerged processes (89, 90, 91, 92). Hromatka & Ebner found that the same amount of substrate could be fermented under conditions of optimum aeration 30 times as fast as in the conventional vinegar towers. Under submerged conditions interruption of aeration for even a few seconds damages the bacteria, hence recognition and prevention of this makes the technical submerged vinegar fermentation possible.

Calcium gluconate has been produced on a limited scale for quite a number of years. It has been shown that sodium gluconate prevents the formation of insoluble precipitates in waters to which caustic soda has been added. This is of considerable interest to companies using automatic bottle washing machines. Recently the Northern Regional Laboratories and the Diversey Corporation of Chicago (24) collaborated in pilot plant studies on the production of sodium gluconate directly, employing a process of continuous neutralization of the gluconic acid produced by *A. niger* at a controlled pH. Factors affecting production which were investigated include antifoam agents, sterilization methods, type and volume of inoculum, agitation and aeration rate, sugar concentration, and depth of medium. Recovery procedures and cost estimates are also given, and it was demonstrated that sodium gluconate can be successfully produced in submerged fermentation.

Vitamins

The biosynthesis of vitamins and the development of an extensive fermentation industry for their production, especially for feed supplements, is comparatively recent. Most of the known vitamins are produced synthetically, but the organic chemists are encountering increasing competition from the microbes, particularly in the case of riboflavin, and more recently in the preparation of vitamin B_{12}.

Riboflavin.—The microbial synthesis of riboflavin was reviewed by Pridham in 1952 (172). He has presented an excellent picture of the various sources of this vitamin and lists 214 references and patents granted mostly within the past decade. Practically all of the intensive studies made on the different industrial processes have appeared since 1947. No attempt will

be made here to survey the patent literature, but the results of many laboratory and pilot plant investigations are now available, and these should be mentioned.

Much of the work prior to 1947 was done either with *Eremothecium ashbyii* or on the recovery of high riboflavin feed concentrates from the acetone-butanol fermentation residues. In 1936 Wickerham and co-workers (228) described a variant strain of a culture of *Ashbya gossypii* which had been in their collection at the Northern Regional Laboratory since 1943. Pridham & Raper (171) studied the taxonomy and relationship of the organism to other fungi and described its cultural, morphological, and physiological characteristics. This variant, in contrast to other strains of the fungus, appeared quite promising for the production of riboflavin. Other groups of investigators in the same laboratory began work with the organism and have since developed one of the most promising industrial fermentations now known for riboflavin production. Yields up to 1760 μg. of the vitamin per ml. of medium in shake-flask cultures were attained through intermittent feeding of glucose during the course of the fermentation (169). Prior to this another patent had been issued to Tanner, Wickerham & Van Lanen (202). Tanner *et al.* (203) also studied the production of riboflavin in submerged aerobic fermentation, paying particular attention to the type and concentration of protein and carbohydrate sources. They concluded that a combination of corn steep liquor and animal stick liquor was best, with either glucose, sucrose, or maltose. Small quantities of a young culture for the inoculum, minimum sterilization of the media, and efficient aeration were also important. The next year Pfeifer *et al.* (165) described results of semi-pilot plant and pilot plant scale studies using deep tank equipment. Factors investigated included sterilization methods, medium composition, aeration and agitation, temperature of fermentation, and strain variation. Yields of 500 to 848 μg. per ml. were obtained in the best fermentations, and 96 per cent recovery of the riboflavin was obtained from dried concentrates. Pridham *et al.* (170) have recently published a bulletin outlining in detail the methods used for shake-flask fermentations, 50 litre aluminum fermentors, and pilot plant fermentations in 300 to 800 gallon equipment. They stress the fact that the *A. gossypii* fermentation is not adversely affected by the presence of certain inorganic ions as are most of the other processes and that high yields may be obtained using low cost raw material. In 1951, a group in the Hiram Walker & Sons Inc. Laboratories used the high yielding Northern Regional Research Laboratory strain of *A. gossypii* in a study (188) on the upgrading of screened stillage. This substrate, fortified with glucose and animal stick liquor, provided an excellent substrate for the organism, and it was possible to increase the riboflavin content one thousandfold. There was also an increase in vitamin B_{12} and an unidentified chick growth factor.

Recent work with certain species of *Candida* yeasts indicate that they have potentialities for the production of riboflavin in simple synthetic media. Levine *et al.* (128) of the Pabst Brewing Company report yields as high as

567 μg. per ml. with *C. flareri*. The iron content of the medium must be held to a minimum, 40 to 60 μg. per l.

Although *E. ashbyii* undoubtedly still is used by several producers of riboflavin, no recent production studies are available. Dulaney & Grutter (54) have studied its nutritional requirements and find that *i*-inositol, but no other growth factors, is required. In 1951 McClary (137) was granted a patent for production of riboflavin using *Mycocandida riboflavina*.

Vitamin B_{12}.—Since the isolation of Vitamin B_{12} in 1948 by Rickes *et al.* (175) and Lester Smith (126) rapid developments have taken place in the field of microbiology and fermentations relating to the production of this important compound. The fact that it proved to be identical, at least in part, to the elusive factor formerly known as the "animal protein factor" made it a discovery of great significance. Its value in human medicine for treatment of Addison's disease, and its widespread application in nutrition of man, animals, and microorganisms all contributed to the interest which has been displayed by scores of workers throughout the world. Furthermore, since there is little evidence that vertebrates can synthesize this vitamin, and green plant tissues are poor dietary sources, the signs all pointed to intestinal microorganisms or rumen flora as the most important sources of supply. It was, therefore, evident that a biosynthetic approach might lead to a process of production that could supplant liver as the chief source unless it was possible to synthesize B_{12} cheaply. Intensive studies had already been made on the production of animal protein factor supplements (33) by fermentation processes, particularly from residues of streptomycin and aureomycin fermentations. It was at this point that Stokstad *et al.* (198) noted evidence of an auxiliary growth factor for chicks in the *S. aureofaciens* fermentation residues, and shortly afterwards the Lederle group announced the growth-promoting effect of aureomycin on pigs (105) and definite proof of its effects on chicks (199). Actually, a similar growth response with chicks had been noted in 1946 by workers at Wisconsin while using streptomycin and sulfonamides to alter the intestinal flora (141). A flood of reports on the growth promoting properties of penicillin, streptomycin, aureomycin, terramycin, bacitracin, and other antibiotics (46) immediately followed the announcement by the Lederle workers of their experiments. It is impossible to review these here, but the subject has been mentioned because it is almost inextricably interwoven with the basic studies on the production of and responses to Vitamin B_{12}. In many respects this antibiotic effect has tended to overshadow the great fundamental discovery of B_{12} and has probably delayed the working out of its basic role in nutrition.

In contrast to riboflavin, in which a specific fermentation is frequently carried out to produce the vitamin most of the supply of B_{12} is as a recovery product from antibiotic fermentation residues. Few details on the exact methods of recovery have been published to date. Jackson *et al.* (99) have described a process whereby B_{12b} may be obtained as a co-product of neomycin production. The acidified fermentation medium is heated to free

the vitamin from the mycelium, and after various absorption and elution procedures a crystalline product is obtained. Leviton & Hargrove (129) have reported on the synthesis of B_{12b} by the propionic acid bacteria. Apparently high concentrations of the vitamin are stored in the cells of these bacteria, since they obtained 1.2 mg. of B_{12b} from each gram of cells harvested. Hodge et al. (86) have used a mixed bacterial fermentation on a 3 per cent soybean oil meal mash with good results. A pure culture of *Aerobacter aerogenes* is seeded into the slightly alkaline medium to remove the carbohydrates. After 48 hr. of aerobic fermentation the medium is sugar free, and it is then inoculated with a mixed culture under anaerobic and slightly alkaline conditions. B_{12} formation is rapid, and maximum production is obtained within 16 hr. The vitamin which is present in the cells can be recovered after filtering or centrifuging the medium and autoclaving the cells so obtained to release it from them. On large scale production it is possible to acidify the beer, then spray dry it to obtain a high potency, stable concentrate which is nontoxic to chicks. Tarr and his associates in the Fisheries Research Laboratories in Canada (204, 205) have studied the production of B_{12} by many organisms grown on herring press water. *Serratia marcescens* was one of the highest producers, although both *Streptomyces griseus* and *S. aureofaciens* do well on this medium. Burton & Lochhead (28) have surveyed 537 bacterial and 676 actinomycete cultures obtained from soils, seeds, manure, and poultry house litter for B_{12} using the Dorner agar plate *L. lactis* assay. They found that 64.6 per cent of the bacteria and 66.6 per cent of the actinomycetes showed activity. *Nocardia* species from soil gave the highest B_{12} assay with *L. leichmanni*. A very complete survey of B_{12} production from poultry house litter and droppings was carried out by Halbrook et al. (76). The vitamin was produced by yeasts, molds, and many types of bacteria. An *A. aerogenes* strain was the highest producer, being superior to *B. megatherium*, *S. aureofaciens* or *S. griseus* under their test conditions. Hall et al. (77) have screened approximately 5000 strains of molds, yeasts, actinomycetes, and bacteria for the production of B_{12} and concluded that only the actinomyces and bacteria were prolific producers of this vitamin. The final results of their work on the actinomycetes has not been published, although a review was presented at the American Chemical Society meetings in Chicago in 1950. According to Raper (173), Hall's group at the Northern Regional Research Laboratories also developed a process involving growth of *S. olivaceus* on thin stillage which gave B_{12} yields of up to 3 μg. per ml. of medium. It was necessary to add 1.5 p.p.m. of cobalt chloride to obtain maximum yields. Raper also states that Lewis and co-workers in the Western Regional Laboratory have used a selected strain of *B. megatherium* and developed a primary fermentation for production of the vitamin. Numerous other references to papers given at meetings and to patent applications might be cited on B_{12} production, but until more details of these studies become available there is little point in attempting to include them in this review.

BACTERIAL POLYSACCHARIDES

Dextran.—Breweries have long been plagued by *Acetobacter* species which cause "ropy beer." Sugar factories and pulp and paper mills suffer from contaminating slimes, produced mainly by species of *Leuconostoc.* Other than trying to prevent their formation, there was little interest in these polysaccharides until 1945 when Gronwall & Ingleman (74) demonstrated that the slime from *Leuconostoc* cultures could act as a blood plasma extender when partially acid hydrolyzed. Commercial production of the dextran followed in Sweden, England, South Africa, and the United States, and meanwhile many different organizations have studied the production, chemistry, and clinical applications of this polysaccharide.

A timely general review on bacterial polysaccharides was compiled for the Sugar Research Foundation in 1947 (65), in which existing chemical knowledge of the various known compounds was presented. In 1949 Stacey & Swift (193) reported studies on a dextran produced by a new strain of *Leuconostoc mesenteroides.* Molecules of the product showed an unusually high degree of branching as well as greater stability in water solutions. Jeanes *et al.* (103), using *L. mesenteroides* NRRL B-512, found that the development of maximum viscosity in the medium coincided with the end of dextran formation and thereafter the viscosity decreased. They recommended using unaerated media, whereas Koepsell & Tsuchiya (111) preferred mild aeration using the same organism. These authors claimed high yields of dextran on a medium suitable for industrial production. The amino acid, vitamin, and growth factor requirements of *Leuconostoc* strains were studied by Whiteside-Carlson *et al.* (225, 226) in sucrose, glucose, and fructose media. While marked strain differences occurred it appeared that nicotinic acid, thiamine, and pantothenic acid were required with all these carbon sources, whereas biotin was not essential with sucrose. The amino acids required were glutamic acid, valine, threonine, tryptophan and histidine.

Although the first blood plasma extenders were made from acid hydrolyzed dextran, an effort was made to find enzymes which would perform this function. Ingelman (96) reported that an enzyme extract prepared from cultures of the bacterium *Cellvibrio fulva* would break the dextran into large fragments. Hultin & Nordstrom (93) selected species of *Penicillium* and *Verticillium* which hydrolyzed dextran if grown on a dextran-containing medium. This dextranase attacks all 1,6 linkages of the dextran molecule with equal ease and has little or no effect on end groups. Recently Whiteside-Carlson & Carlson (227) have claimed that the main effect of the above enzymes on the polysaccharide is the production of reducing sugars, with the result that they are of no practical value in the preparation of partially degraded dextrans. These authors isolated a species of *Aspergillus* and prepared filtrates which they claimed only produced a small amount of reducing sugar during the time required to split the dextran into fragments with an average molecular weight of 75,000.

The "ropy beer" organisms, *Acetobacter viscosum* and *A. capsulatum,*

are also capable of producing abundant amounts of polysaccharide material with serological properties similar to those of the *L. mesenteroides* dextran according to Hehre & Hamilton (83). Later chemical analysis confirmed their earlier work and showed the dextran to be a high molecular weight polymer of D-glucopyranose units linked principally in the α-1,6 positions (82). These bacteria must be grown on dextrin rather than other common carbohydrates to produce the desired product.

In addition to dextran there are many other bacterial polysaccharides, some important in relation to soil structure, others in medicine. Stacey (194) has published a short general review of the subject.

ENZYMES

The field of enzyme technology is making rapid advances at present, but the greater part of the published work is of a fundamental nature. The industrial laboratories must be making advances in technology, but, to date, companies interested in producing and marketing enzyme products seldom expose their techniques in other than very general articles or the patent literature. A comprehensive two volume work, "Enzymes," was published by Sumner & Myrback (200), and in it the reader will find numerous chapters on enzymes from higher plants, animals, and microorganisms as well as a section on enzyme manufacture and the application of industrial enzymes. To glance over these chapters will indicate the futility of attempting a detailed review of all the advances in microbial enzymes of the past five years. Consequently the writer has limited the topics to be discussed to three, namely the work on amylase, proteases, and the very recent developments concerning the oxidation of sterols by microbiological processes. Recent general papers by Lockwood (131) and Smythe (190) give an interesting account in general terms of certain processes and stress the wide uses of their products in modern industry.

Mold amylase.—Prior to 1947 workers at Iowa State College made intensive studies on the production of amylase preparations obtained by growing *A. oryzae* on wheat bran, Underkofler *et al.* (214) published a detailed account of laboratory and pilot plant studies early in 1947. They studied growth of mold on moist, sterilized wheat bran in rotating drums, in trays, in perforated trays, and in specially designed humidified and ventilated incubators. At one time a commercial plant produced 10 tons a day for use in saccharifying grain mashes for alcohol production and preparing enzyme converted syrups from starch. In spite of advances in the mechanization of tray production most microbiologists believed that it should be possible to obtain satisfactory enzyme production in submerged tank fermentations. After their success with penicillin it was natural that the Northern Regional Laboratory should attempt a program of research with this in view. LeMense *et al.* (119) surveyed 350 fungi for their ability to produce amylase when grown under submerged aerobic conditions. Of the Aspergilli tested, strains of *A. oryzae, A. alliaceus* and *A. wentii* produced substantial amounts of

dextrinizing enzyme, whereas *A. niger* NRRL 337 made both dextrinizing and saccharifying enzymes when grown for three to five days in aerated thin stillage plus 2 per cent corn meal and 1.5 per cent calcium carbonate. In co-operation with Joseph E. Seagrams & Sons it was found that this enzyme preparation could be used to replace barley malt completely for industrial alcohol production from grains (1). During the next three years further studies were reported from the Peoria laboratories (62, 120, 213) on factors affecting the production of alpha-amylase and maltase by *A. niger*. They indicated that some measure of control of the yields of these enzymes could be obtained. Pilot plant studies provided comparable cost data with malt for alcohol production. At the Prairie Regional Laboratory at Saskatoon, Shu & Blackwood (182) were able to vary the ratio of maltose and alpha-amylase by adjusting the carbohydrate and nitrogen sources and the fermentation time. Later Shu (184) found that proteins or amino acids were not necessary for high yields but, where appreciable transient acids were produced, the use of a nitrogen source which was a potential alkali donor greatly increased the yield of alpha-amylase.

Proteases.—Lennox (123) has recently reviewed the properties and uses of various proteolytic enzymes, including those produced from various bacteria, yeasts, and molds. Maxwell (136) tested 43 strains of molds on steamed wheat bran, and she found that *Aspergillus flavus-oryzae* produced the greatest amounts of enzymes with greatest gelatinase activity. Crewther & Lennox (45) were the first to prepare a crystalline enzyme from a mold culture. They grew *A. oryzae* on a liquid medium in still cultures and, after processing the medium, obtained needle-shaped crystals, mostly in the form of rosettes. This enzyme ranks with trypsin as the most active enzyme for digesting gelatin. Studies have also been reported from Japan on the preparation of mold enzymes. Yuki (235) used a crude prepartion from *P. notatum* and studied the reaction on gelatin, casein, and peptone. He concluded that that protease from this mold was different from that of animal and plant sources.

In our laboratory Dion (49, 50) studied 289 cultures of fungi and actinomyces for protease production in submerged culture. Twenty different organisms were found to produce moderately high yields of proteolytic enzymes under the conditions used. Powdered milk and malt sprouts provided the best media for protease production. McConnell (138) has studied some of the characteristics of these enzymes and found that they resemble trypsin in that they are more active at a pH above seven and are inhibited by a preparation of egg albumin. Wetter (223) has since attempted to purify some of these mold enzymes. Working with the one produced by *Mortierella renispora*, because it appeared to be the least complex, he found two electrophoretically distinguishable proteins with proteolytic activity. The two enzymes differed in heat stability, isoelectric points, and solubility properties, one being albumin-like and the other globulin-like. The pH optimum appears to be the same for both.

Microbiological oxidation of sterols.—No review of enzymes today can afford to overlook the recent developments which have taken place in the manufacture of cortisone using microbiological oxidation to introduce oxygen on the 11 position in the precursor, thus saving numerous steps in the synthesis. Actually the original work in this field goes back to 1937, when it was observed that steroid alcohols were converted to ketones by organisms. Horváth & Krámli (87, 88) studied the microbiological oxidation of cholesterol with *Azotobacter* and reported on their results in 1947. They were able to transform cholesterol to cholestenone. The organism dehydrates within the sterol ring making it possible to isolate 7-dehydrocholesterol. At that time other workers suggested that alkaline conditions brought about the changes rather than activity of the organism. These authors refuted this argument in a further paper which appeared in 1949 (88).

The work of Peterson & Murray (162) on the microbiological oxidation started an interesting series of investigations in a number of different companies to find a short cut in the synthesis of cortisone. These authors used a species of *Rhizopus* and brought about an oxidation of the steroid progesterone at carbon 11. The fermentation was carried out for 24 to 48 hr. in a lactoalbumin digest-dextrose-cornsteep medium. By this means a new 11-oxygenated steroid intermediate became available for conversion to cortical hormones. Similar microbiological oxygenations at carbon 11 were made on androstenedione, 11-desoxy-17-hydroxycorticosterone and 11-desoxycorticosterone. Another group of workers from the University of Illinois, Lilly Research Laboratories, and Biochemical Institute of the University of Texas (39) described a partial microbiological synthesis of adrenal cortex hormones and presented evidence for the microbiological oxidation and conversion of Reichstein's Compound S to Kendall's Compound F by *S. fradiae.* Other workers from the Squibb Laboratories (69) made a systematic study of the metabolic action of a variety of organisms and a number of sterol substrates. They found that *Aspergillus* species are capable of effecting the desired changes in practicable yields. Several new compounds were found. One from Reichstein's Compound S proved to be easily converted to cortisone acetate in 70 per cent yield. Perlman *et al.* (159) from the same laboratory found that an unidentified actinomycete brought about the introduction of one or more hydroxyl groups into the intact steroid nucleus of progesterone. The microbiological conversion of pregnenolone to progesterone using various actinomycete and mold cultures (161) has also been reported. This work confirmed earlier studies made by Mamoli using bacteria. Large scale production using these techniques for conversion of steroids has already been undertaken by various pharmaceutical companies.

FOOD AND FEED

Much has been written in recent years about shortages of food which are likely to take place if the world population continues to increase. Few of these articles have taken into account the potentialities of microorganisms

for converting simple compounds or waste organic materials into more complex proteins, fats, or carbohydrates, which with suitable processing could then be used as sources of food for humans or livestock. A start has been made on the investigation of these problems in different parts of the world, but only a few of the potential microorganisms have been assayed for their usefulness. Two general articles which review a few of the possibilities are those of Yin (234) and Meier (140). In considering this problem one should bear in mind that there are two large classes of microorganisms; those containing chlorophyll, capable of carrying out photosynthesis and converting inorganic compounds into organic matter, and the nonphotosynthetic bacteria, fungi, and actinomycetes, capable of transforming one form of organic material to another and possibly more desirable form. The microbial synthesis of fats, while uneconomical at present, is of potential significance. A good general review of the subject was presented by Hesse (84). Pan *et al.* (153) have demonstrated that *Rhodotorula gracilis* may be grown successfully in a molasses medium and produce fat with the same efficiency as on special glucose media. The Swedish investigators, Nielsen & Nilsson (149) have investigated the respiration, growth, and fat production of the above yeast, grown on different carbohydrate sources. They emphasized the conversion of pentose sugars to fat because of their interest in the utilization of wood hydrolyzates.

Probably the most intensive studies to date have been made with yeasts, or yeast-like fungi for protein and B-vitamin supplements. Although German scientists were among the first to realize their possibilities, investigators in the United States have published much of the recent work. Atkin *et al.* (9) using brewer's yeasts, have studied their growth and fermentation characteristics, as well as nutrient requirements. During the past five years members of the Department of Biochemistry at the University of Wisconsin (2, 3, 32, 151, 186) have evaluated the potentialities of four different organisms, *Saccharomyces cerevisiae, Torulopsis utilis, Candida arborea,* and *Oidium lactis.* Numerous factors affecting the fermentation and yields of product on different substrates were investigated, as well as the vitamin requirements and the essential components of synthetic media. In the Forest Products Laboratory at Madison (79) both continuous and batch fermentation studies on the growth of Torula yeast on wood hydrolyzates has received attention. Studies also were made on the growth of this organism on waste sulphite liquor by the Sulphite Pulp Manufacturing Research League (230), and later a combined staff report by this organization and Industrial and Engineering Chemistry summarized processes and possibilities in production of food yeast (97). They concluded that the success of food yeast production was more a question of economics and product promotion than of fundamental fermentation technology. Most of the difficulties in the latter field have now been eliminated.

Humfeld (94) has studied the production of mushroom mycelium in submerged culture. It was possible to produce a mushroom mycelium under these

conditions which was quite comparable chemically to that of the natural fungus.

Recently a number of reports have appeared on the production of algae for food. The large amount of work now being done on photosynthesis with algal cultures has stimulated further research on the potentialities of these green microorganisms. Cook of the Stanford Research Institute (40) has developed a laboratory scale continuous process for growing *Chlorella pyrenoidosa* in both artificial light and natural sunlight. Many different factors were investigated and the process is now at a point where it merits further large scale testing.

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MICROBIOLOGY OF WATER AND SEWAGE[1]

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Intestinal Bacteria

Enumeration.—An advance has been made in the technique of enumeration of intestinal bacteria by the development of membrane filtration (1, 2, 3, 4, 5). The method has advantages over the standard method for the determination of coliform organisms in potable and polluted waters because it allows a concentration of a small number of organisms from large quantities of water, and a reduction in time, labor, material, equipment, and space. The method is accurate, reliable, and direct. By utilizing special differential media the method can be applied to isolation, identification, and enumeration of bacteria other than the coliform organisms.

Survival and viability.—Extensive studies made on the effect of storage on water samples showed a significant increase of coliform organisms in 15 per cent of 151 samples stored overnight at room temperature (16° to 23°C.) and in 7 per cent of the samples stored in a refrigerator (2° to 5°C.). Significant decreases occurred in 19 per cent of the samples at room temperature and in 17 per cent of the refrigerated samples (6).

Various members of coliform groups remain viable for long periods upon storage of inoculated autoclaved and nonautoclaved estuarine waters. The numbers manifest cyclic changes (7). In acid mine waters *Escherichia coli* dies rapidly, but a small number of cells survive after 24 hr. at pH 2.0 (8).

In evaluating the factors contributing to the decrease of coliform organisms in tidal estuaries, dilution, bactericidal action of the sea water, and the predation by zooplankton are sufficient to account for nearly all of the decrease. In the river end of the estuary the effect of sea water is not much greater than that attributable to dilution of the fresh water. As the water moves down the estuary the bactericidal action of the sea water is greatly increased. The effect of predation also increases as the water moves down the estuary. The decrease in coliform bacteria not accounted for by the combined action of these three factors should be attributed to sedimentation (9).

E. coli was demonstrated to be adsorbed on silts obtained from rivers and estuaries. The degree of adsorption is influenced, among other things, by the character of the silt and is probably a function of the base exchange capacity and electrical charges of the silt particles. The adsorption of *E. coli* on silt particles may be of prime importance in determining its rate of sedimentation in natural water (10).

Pathogenic organisms.—Repeated isolations of Paratyphoid bacilli were made by the swab method from river water (11). *Salmonella typhosa* was isolated from a river water and the source of the organism was traced to a carrier (12). *S. typhosa* and *S. paratyphi B.* were identified from processed sewage effluent and from the river water below the point of discharge of the effluent (13). A significant number of *Salmonella* were isolated from sewage-contaminated irrigation water. Vegetables irrigated with such waters, however, did not contain these organisms (14). Several types of *Salmonella* were isolated from irrigation water. The median value of 11 samples was 0.9 cells of *Salmonella* per 100 ml. of water (15). A new *Salmonella* type was recovered from a river water. Accidental ingestion of the organism was followed by enteritis and diarrhea (16). An outbreak of gastroenteritis affecting a considerable number of people in a factory was reported. The examination of the drinking water showed more than 180 coliform organisms per 100 ml. *Shigella sonnei*, *S. flexneri* and *S. typhimurium* were isolated from the fecal specimens of the affected people (17).

Enterococci can be used as good indicator organisms of fecal contamination from the standpoint of public health hazards from sewage in soils and vegetables. These organisms die more rapidly in soils than coliforms, while typhoid bacilli die more rapidly than enterococci. The longevity of all these bacteria increases with the organic matter content of the soil (18).

A comparison of enterococci and *E. coli* in polluted salt water revealed that the enterococci densities followed the pattern of *E. coli* indices, but at a lower magnitude. The *E. coli* density averaged about 63 times that of the enterococcus density (19).

A comprehensive study to determine the health hazard involved in the consumption of raw vegetables raised in polluted soils revealed that coliform organisms did not exist in higher numbers on tomatoes grown on sewage-irrigated soils than on those grown on soil which had not received sewage. After spraying suspensions of *E. coli* or feces on the growing tomatoes, the number of coliform organisms decreased to or below the level of uncontaminated controls within 35 days. If the source of contamination is stopped within a month before harvesting of the tomatoes, the transmission of enteric bacterial disease through the consumption of raw tomatoes will be negligible. The survival of *Salmonella* and *Shigella* on the tomato surfaces did not exceed seven days, even when the organisms were applied with fecal organic matter (20).

Endamoeba histolytica cysts applied directly to tomatoes and leaf lettuce, or to soil in which the plants are grown, died rapidly as a result of desiccation. The drying of the cysts resulted in instantaneous death. Organic matter in the form of fecal suspension did not give protection to the survival of the cysts. A period of 1 to 2 weeks between the last application of polluting material and harvesting is sufficient to render safe the raw consumption of these crops (21).

Ascaris eggs sprayed on tomatoes and lettuce growing in the field per-

sisted for over a month. However, the eggs had degenerated and were incapable of development for infection (22). The storage of harvested contaminated tomatoes does not afford sufficient protection, as the death rate of coliform organisms under these conditions is slow. The most effective method of decontamination of such crops is by soaking them in water at a temperature of 60°C. for 5 min. Such treatment does not affect the appearance of the tomato (23). For the decontamination of *Ascaris* infected tomatoes the same procedure of soaking in water at 60°C. for 10 min. is an adequate and safe protective measure (24).

The normal habitat of *Pseudomonas aeruginosa* was found to be in human intestines; the organism can be isolated consistently from sewage (25).

An epidemic of acute infectious hepatitis occurred in a rural area in Pennsylvania. The well water on the farm was found to be fecally contaminated from a nearby cesspool (26). Another investigation of an epidemic hepatitis gave evidence that the water supply was the source of infection (27).

Virulent tubercle bacilli were recovered by guinea pig tests from sewage of tuberculosis sanitoria and the streams receiving the sewage from these sanatoria (28). Seven samples of sewage effluent from tuberculosis hospitals showed the presence of tubercle bacilli. Tubercle bacilli in a ground suspension of infected guinea pig organs placed in collodion bags and suspended in streams remained viable as long as 48 days (29).

Antibiotics and bacteriophage.—A bacteriophage for *Clostridium welchii* was isolated from polluted water in quantities proportional to the degree of pollution (30).

Enterobacteriaceae in polluted waters decreased progressively for the first few days and then dropped abruptly on the seventh, accompanied by a rapid clearing of the medium. No clearing was observed in autoclaved waters. Two *Salmonella* phages were isolated from the waters, one of which was specific and the other multivalent (31).

Strains of *Bacillus subtilis* and *B. cereus* were isolated from well waters which were antagonistic to *E. coli*. The *B. subtilis* strains also produced antibiotic substances that were active against *S. flexneri*, *S. typhosa*, *S. paratyphi*, and *Micrococcus pyogenes*. It is suggested that the presence of *Bacillus* species may affect the longevity of enteric organisms in surface waters (32).

An antibiotic was extracted from *Microcystis aeruginosa* which had an antibacterial effect on *Clostridium bifermentans*. Filtered pure cultures of *Scenedesmus* have a similar effect on *C. sporogenes* (33).

The rapid disappearance of coliform bacteria from normal sea water under laboratory conditions is attributed to a bactericidal property. The nature of this bactericidal factor has not been identified, but it is suggested that it might be antibiotic substances produced by marine organisms, bacteriophage, or autolytic products of the coliform organisms themselves. This suggestion is based upon the fact that the bactericidal factor is thermolabile (34).

Disinfection.—The relative resistance of *S. typhosa* and *E. coli* to the

bactericidal action of free available chlorine depends on the pH and amount of chlorine. At pH 7.8 or less, and at a free available chlorine content of 0.03 p.p.m. or less, *S. typhosa* is slightly more resistant than *E. coli*, while at all other concentrations of chlorine at pH 7.8 or less, and at pH 8.5 or greater, the reverse is true. Combined available chlorine is much less efficient as a bactericidal agent than free available chlorine. The data indicate that coliform groups are safe indicators for the determination of the potability of water, and minimum chlorine residuals proposed have a considerable safety factor to kill the most resistant species (35).

Plant scale experiments on the disinfection of coliform organisms in sewage with chlorine showed the importance of proper mixing of chlorine with sewage. Important also are contact time and quantity of residual chlorine. When residual chlorine content is low, contact time becomes even more important (36).

That the discharge of chlorinated sewage into a receiving body of water might give rise to an aftergrowth of coliform organisms was demonstrated by laboratory experiments. When chlorinated sewage was diluted with unpolluted fresh water there was a significant increase in the numbers of coliform organisms after 1 day. The increase was greater with higher concentrations of sewage. An increase of coliform organisms did not take place when chlorinated sewage was diluted with sea water (37).

Inconsistencies in bacteriological results were obtained in chlorinated sewage from a number of treatment plants. With identical chlorine residuals variable numbers of coliform bacteria were found to survive (38).

Elemental iodine is an efficient disinfectant of water. Its action is similar to that of chlorine. Like chlorine its action is greater at higher temperatures and lower at high pH values (39).

In lime treated waters with high pH values (11.0 to 11.5) *E. coli*, *S. typhosa* and *S. montividio* do not survive (40).

Significant reduction in coliform organisms is obtained by ozonation of raw surface waters with high turbidities. An average reduction of 99.4 per cent of coliform organisms was obtained (41).

Ultrasonic energy (400 kc.) is effective in reducing the numbers of coliform bacteria in aqueous systems. The age of the culture is an important factor in the germicidal action of the ultrasonic waves, the younger cultures being more resistant than older ones. The germicidal rate is independent of the initial concentration of bacteria over a wide range. Temperature has an important influence on the killing rates of ultrasonic waves (42).

PLANKTON ORGANISMS

When organisms are used as indicators of water quality, the whole biological community must be considered (43). Protozoa alone are not good indicators of pollution, but protozoa along with other animals can serve as good indicators of pollution. Protozoa are sensitive to toxicants and therefore can

be used to good advantage as bioassay organisms in the detection of toxic wastes (44). The evaluation of the effects of industrial pollution can be better achieved by studying the fish habitats and their productivity rather than by physical and chemical examination of the wastes or the receiving waters, or by observing indicator organisms. Studies of bottom fauna and plankton are very instructive (45).

A study of the algal flora of a London reservoir showed that as a result of closing the inlet to the reservoir and the subsequent stagnation of the water, marked chemical and biological changes occurred (46).

Earthy odors produced in a river water were traced to the growth of *Streptomyces* in the river bottom sand exposed to drying (47). A study of the distribution of fungi in polluted zones of a river showed that some species were always absent from heavily polluted waters and others were found in moderately polluted areas. *Aphanomyces* showed an affinity to polluted matter (48).

The production of oxygen by algae in daylight generally exceeds the consumption during the night, but when a large proportion of sewage is present the dissolved oxygen disappears completely both during daylight and darkness (49). Under favorable climatic and other conditions, sewage ponds afford a simple and economical means of biological stabilization of sewage and industrial wastes. The treatment in these ponds results from a complex symbiosis of bacteria and algae. Bacteria oxidize the organic matter in sewage to carbon dioxide. The algae through photosynthesis convert the carbon dioxide to algae cell material and in turn furnish oxygen for the oxidation of the organic matter by bacteria. The effluent from the pond may, therefore, contain as much organic matter as the influent to the pond, with the difference that while the influent is highly putrescible, the algal cells in the effluent are stable and may be of value in the stream in promoting fish life. *Euglena gracilis*, under controlled experimental conditions in the presence of abundant food and light, is small, dark green, highly motile, reproduces rapidly, contains a minimum of fat, and produces more oxygen than it respires. Algae absorb ammonia and other forms of nitrogen from sewage, converting them to cell protein which may be separated, recovered from sewage, and used as cattle food (50).

Algal toxicity.—Toxic plankton in water have caused livestock losses (51) and may be the cause of unexplained dysentery epidemics similar to the condition known as Haff's disease in Europe (52). A number of other cases of livestock poisoning caused by the poisonous algal blooms in water have been reported (53, 54). Domestic animals died upon drinking water blooming with *Aphanizomenon floc-aquae*. The water from which this organism was removed was nonlethal. The growth of poisonous algae is stimulated by continuous hot weather and high organic matter contributed by sewage pollution. Fresh algae are more toxic than decomposed algae (55). Frozen and dried algae are most toxic. The chemical nature of the algal toxin is unknown, but it is claimed to be secreted into the surrounding medium.

Poisoning of livestock in South Africa by a blue-green algae *Microcystis toxica* is reported (56). The toxin was isolated and identified as an alkaloid.

MICROBIOLOGICAL PROCESSES IN WASTE DISPOSAL

Processes related to water, sewage and wastes.—Bacterial corrosion of concrete sewers is dependent upon the following chain of events: (*a*) production of hydrogen sulfide from sewage; (*b*) emission of hydrogen sulfide from the sewage into the sewer atmosphere; (*c*) fixation of the hydrogen sulfide by the concrete sewer walls; (*d*) bacterial oxidation of the adsorbed sulfide into sulfuric acid; and (*e*) attack of the acid on the concrete wall (57). The utilization of hydrogen in the reduction of sulfate by a number of cultures of *Vibrio desulfuricans* was demonstrated and evidence was obtained that hydrogenase is an adaptive enzyme (58). A new autotrophic iron-oxidizing bacterium, *Thiobacillus ferrooxidans* was isolated from acid mine water. The organism grows at low pH values, which precludes the chemical oxidation of the iron by atmospheric oxygen. This organism can also grow autotrophically on thiosulfate (59).

Radioactive phosphorus (P^{32}) when present in a concentration of 10 millicuries per liter exerts no significant effect on the rate or quantity of biochemical oxygen demand of sewage (60). The retardation of oxidation of polluted waters by small amounts of cyanide is variable depending upon the type of water and microorganisms present. With a specialized inoculum the oxidation of organic matter occurs in the presence of cyanide after an initial lag period, and the cyanide content of the water decreases. Smaller doses of cyanide have a proportionately greater effect on oxygen utilization (61).

Phenol and *o*-cresol undergo a complete destruction under anaerobic conditions similar to those encountered in some streams and lagoons. The rate of destruction is, however, considerably slower than under aerobic conditions. It follows that in a stream which is devoid of oxygen these materials will persist longer and be carried further downstream than in streams in which oxygen is present (62).

In order to find new methods for the utilization of waste wood, a thermophilic culture of bacteria was developed which was able to convert considerable portions of different kinds of wood to acetic, butyric, and lactic acids (63). A review of the literature revealed no evidence of bacteria or enzymes capable of bringing about the degradation of lignin (64).

Sisal, manila, hemp, flax, jute, artificial silk, nylon, and perlon fibers subjected to bacterial action under aerobic and anaerobic conditions were decomposed. Natural fibers decomposed within 2 to 3 years and artificial fibers within 3 months to $2\frac{1}{2}$ years (65).

Aerobic processes for the treatment of sewage and wastes.—The organisms found in trickling filters have been classified as follows (*a*) filter film organisms; (*b*) film surface organisms; and (*c*) filter cleaning organisms. The dissolved organic matter in sewage is said to be adsorbed biologically by the

filter film organisms for assimilation and stabilization, while the suspended and colloidal solids are adsorbed physically on the film surface first and then utilized by the film surface organisms especially protozoa (66). In an under-loaded trickling filter there is a definite zonal distribution of organisms. With increasing loads, polysaprophytic and mesasaprophytic organisms move downward towards the bottom of the filter (67). A study of the general heter-otrophic bacteria, zoogleal bacteria, and coliform bacteria in the film coating the sand particles used for treating sewage by intermittent sand filtration process was made. Of the heterotrophic bacteria isolated, *Flavobacterium* and *Bacillus* predominated. Zoogleal bacteria were found in the upper layer of the bed, but not in the lower portions. The number of coliform bacteria decreased with increasing depth in the filter. Attempts to show that the decrease of enteric bacteria results from the presence of antibiotic-producing microorganisms met with failure (68).

Protozoa play an important role in the removal of bacteria from sewage by the activated sludge process after the bacteria are adsorbed by the acti-vated sludge floc. Artificial additions of pure cultures of bacteria increase the number of protozoa in the activated sludge (69). Five floc-forming bacteria other than *Zooglea ramigera*, which is commonly associated with the formation of activated sludge floc, were isolated from activated sludge. These included *Escherichia intermedium*, *Paracolobacterium aerogenoides*, *Nocardia actinomorpha*, *Bacillus cereus*, *Aerobacter aerogenes*, and a bacteri-um belonging to the genus *Flavobacterium* (70). A theory is proposed that bacterial flocculation of impurities in sewage by the activated sludge process is brought about by reducing the electrical charge below a critical level and making contact with the cells through agitation. Bacterial surfaces are as-sumed to undergo direct chemical reactions, thus forming a solid bond between the cells. This explanation of a complex phenomenon is not complete but is an attempt to analyze one of the factors involved in floc formation (71).

Skim milk at a concentration of 1000 p.p.m. was completely removed from solution by activated sludge, 60 per cent of it being assimilated into cell tissue and the balance oxidized to carbon dioxide and water (72).

Certain industrial wastes have nitrogen and phosphorus deficiencies for adequate biological treatment by the activated sludge process; correction of these deficiencies by artificial additions of these elements, permits ade-quate treatment of these wastes. In case these wastes are mixed with sewage, the nutritional requirements can be supplied by the excess nutrients con-tained in the sewage (73).

The efficiency of biochemical oxygen removal by activated sludge is a function of the total quantity of sludge present and of the applied load (74). In the purification of sewage by activated sludge, an accumulation of sludge takes place. Attempts made to evaluate some of the factors affecting the growth and accumulation of sludge showed that the quantity of sludge employed in the treatment process had an important effect on the quantity of solids accumulated, being lower with increased quantity of sludge used

in the process. In addition, the biochemical oxygen demand of the sewage fed affected the growth of the sludge (75).

In the determination of the oxygen demand of wastes, direct measurement of oxygen utilization by the Sierp apparatus or Warburg respirometer may be used in some studies more conveniently than by the more conventional Winkler method. Direct methods give similar results (76). The use of these direct methods has made it possible to study experimentally the effect of a number of variables on the rate and quantity of oxygen required in the aerobic stabilization of waste. Oxidation rates of a number of wastes were shown to be independent of hydrogen ion concentration between pH values of 6 and 8. The optimum pH range was wider for some wastes than for others. The effect of pH was more pronounced and the range was more restricted during shorter periods of incubation. The pH values tended to change towards neutrality from both the acid and alkaline sides during oxidation of wastes. Nutritional requirements and deficiencies of nitrogen and phosphorus could be determined readily by the direct methods. For wastes contaminated with bacteria, seeding with sewage did not increase the rate of oxidation; for wastes deficient in microorganisms seeding with sewage was of value. With proper adaptation, the rate of oxidation of materials like phenol could be increased so that 1000 p.p.m. of phenol could be oxidized in 3 to 4 days (77).

For the purpose of developing biological growths with adsorptive properties for the removal of radioactive substances from waste liquors deficient in organic materials, the production of activated sludge from organic materials such as flour and dextrose was studied (78).

Isolation of vitamin B_{12} from activated sludge was reported. From 3.5 to 4.0 mg. of vitamin B_{12} per kg. of dried activated sludge was obtained. Part of the vitamin was found to be derived from raw sewage, and part was synthesized by microbiological action during the aeration of sewage with activated sludge (79).

Anaerobic digestion process.—Two additional methane-producing organisms were isolated and studied. *Methanobacterium suboxydans* converts valeric acid to propionic and acetic acids by means of beta oxidation, and converts butyric and caproic acids to acetate. *M. propionicum* oxidizes propionic acid to carbon dioxide, which arises from the carboxyl group, and acetate, which arises from the alpha and beta carbons. The methane in these fermentations is derived from carbon dioxide (80). Tracer studies showed that in the fermentation of acetate by *Methanosarcina*, methane is derived from the methyl group, and the carbon dioxide from the carboxyl group. Methane fermentation of acetate differs from that of most other substrates in that carbon dioxide reduction plays a minor role in the production of methane. Carbon dioxide reduction is also a relatively minor process in the fermentation of methanol to methane by *Methanosarcina* (81).

The addition of nitrate to a medium in which acetate is being converted to methane by a purified culture results in denitrification to nitrites and nitrogen gas, and complete disappearance of methane from the gas (82).

Sodium sulfite at a concentration of 100 p.p.m. has no effect on the methane fermentation of sodium acetate, but at 500 p.p.m. it has a distinct retarding effect. The effect is bacteriostatic rather than bactericidal. At higher pH values, a greater concentration of sulfite was required to cause inhibition. Calcium sulfite had an effect similar to, though less pronounced than that of sodium sulfite (83). Sodium sulfide at 320 p.p.m. concentration (as S) had no effect on methane fermentation of calcium acetate, but at 1600 p.p.m. inhibited it. Sodium sulfate at 1260 p.p.m. reduced gas production by 70 per cent but had no effect at 126 p.p.m. concentration (84). Soluble sulfides up to 300 p.p.m. (as S) had no effect on the production of volatile acids during the anaerobic digestion of white water, but even 80 p.p.m. retarded methane fermentation of sodium acetate, and at 150 p.p.m. methane fermentation was completely inhibited (85). Sulfates have been shown to have a retarding effect on the anaerobic digestion of wastes. An unbalance of bacterial activity results from the addition of large quantities of sulfate and causes a rapid rise of volatile acids (86, 87, 88).

In the anaerobic digestion of sewage sludge only three volatile acids are produced, namely, acetic, propionic, and butyric acids. These acids accumulate initially when they are not being converted into methane. When methane fermentation becomes active the quantity of these acids decreases (89). In the anaerobic digestion of several industrial wastes, the volatile acids produced consisted of the three acids named above plus valeric acid (90). The concentration of alcohols, acetone, and acetaldehyde produced during the anaerobic digestion of sewage sludge and industrial wastes was low. Even *Clostridium acetobutylicum* produced only a small amount of acetone and alcohol when inoculated into sterilized sewage sludge, indicating that the composition of the substrate is responsible for the small quantities of these substances produced during the digestion. Under anaerobic conditions the digestion proceeds with the intermediate production of lower volatile acids which in turn are utilized by methane bacteria (91).

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